THE ROLE OF PROSTAGLANDINS IN THE MATURATION AND FUNCTION OF HUMAN MONOCYTE DERIVED DENDRITIC CELLS

By

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THE ROLE OF PROSTAGLANDINS IN THE MATURATION AND FUNCTION OF HUMAN MONOCYTE DERIVED DENDRITIC CELLS

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Dendritic cells (DC) are important mediators of immunity and tolerance. Prostaglandins, especially prostaglandin E2 (PGE2), have diverse affects on the adaptive immune response including in vitro maturation and function of monocyte derived dendritic cells (MDC). Using an established protocol for generation of MDC, the role of prostaglandins in the maturation and function of MDC was investigated. This study demonstrates that MDC constitutively express cyclooxygenase-2 (COX-2) during differentiation from monocyte precursors and produce prostaglandins that autoregulate expression of CD83, a mature DC specific marker, and secretion of interleukin-12 (IL-12), a critical proinflammatory cytokine responsible for Th1 immune responses. Interestingly, the effects of PGE2 are highly dependent on the maturation stage of the MDC. In immature-MDC (I-MDC), the presence of PGE2, whether endogenously produced or added to cultures, increases the secretion of IL-12 while in maturing MDC,

PGE2 profoundly decreases the secretion of IL-12. PGE2 mediates its response in MDC through prostaglandin receptors EP2 and EP4, which are members of the G protein-coupled receptor family. EP2 and EP4 stimulate adenylate cyclase which increases cAMP in response to ligand binding. This study finds that I-MDC express mRNA for EP2 and EP4. As MDC mature, the expression of mRNA for EP2 gradually declines by 50% at 24 hours and remains decreased after 48 hours while the mRNA for EP4 increases four fold at two hours and remains significantly increased in the fully mature MDC. Pharmacological agents that target specific prostaglandin receptors show that increases in IL-12 in the I-MDC are mediated through EP2 and EP4 and downregulation of IL-12 in the maturing MDC is mediated through EP2 and high cAMP production. Fully matured MDC produce lower levels of cAMP in response to PGE2, have fewer PGE2 binding sites, and are resistant to modulation of IL-12 by PGE2 as well as cAMP analogues. These findings have important implications for the development of the MDC for immunotherapy as well as the effects of COX inhibitors or selective prostaglandin receptor agonists on immune function, and may provide new approaches to modulation of the proinflammatory immune response.

CHAPTER 1
INTRODUCTION

Dendritic cells (DC) are the most potent antigen presenting cells (APC); they are 10-100 times more potent at activating naïve and memory T cells than B cells or macrophages (MΦ). Constitutive high expression of costimulatory molecules, CD80 and CD86, and major histocompatibility (MHC) molecules make DC unique in their ability to activate naïve T cells (Steinman, 1991). Recently, two subsets of DC have been phenotypically described, a myeloid derived DC that captures antigen in the periphery and migrates to the draining lymph node and a lymphoid DC that resides in the lymph node. Functional differences in the two subsets continue to headline the DC literature (Shortman and Caux, 1997; Steinman *et al.*, 1997a). DC have been shown to be important in both immunity and tolerance. The ability of DC to induce T cell activation or tolerance is dependent on the microenvironment during antigen capture and the antigen itself (Kalinski *et al.*, 1999a; Steinman *et al.*, 1997b). Antigens that fail to induce an inflammatory stimulus are considered safe and induce tolerance while antigens that are accompanied by an inflammatory signal elicit an immune response directed at antigen elimination (Finkelman *et al.*, 1996). Activation of T cells by APC requires T cell receptor (TCR) recognition of peptide presented in MHC molecules and co-stimulation of CD28 on the T cell by CD80 or CD86 on the same APC. Lack of costimulation in the presence of TCR engagement results in anergy of the T cell and presumed tolerance of the antigen.

Because of their central role in the adaptive immune response, DC have become a favorite target for research in many clinical diseases involving T cells including allergy, transplantation, autoimmune disease, resistance to infection, resistance to tumors and immunodeficiency. In autoimmune disease, defects in stimulatory capacity of the DC may promote autoimmunity by impaired antigen presentation that leads to accumulation of autoreactive T cells or deficient generation of regulatory cells. Impaired or suboptimal T cell activation may be sufficient to induce T cell proliferation but not strong enough to induce a tolerogenic or protective response, as generation of regulatory cells and elimination of activated T cells requires a quantitatively higher level of activation than what is required for T cell proliferation (Serreze *et al.*, 1993). In human autoimmune insulin dependent diabetes (IDD), defects in APC function have been described. Monocyte derived DC (MDC) from subjects with IDD generated by triidothronine show reduced ability to cluster and stimulate autologous and allogeneic T cells in vitro (Jansen *et al.*, 1995). Recently, Takahashi et al. (1998) showed that immature MDC (I-MDC) from subjects at high risk for IDD expressed quantitatively lower levels of CD80 and CD86 per cell than age/sex/human leukocyte antigen (HLA) matched controls (Takahashi *et al.*, 1998). Studies in the murine model for diabetes, the non-obese diabetic (NOD) mouse, also suggest that DC play a protective role in the autoimmune process by activation of regulatory T cells (Clare-Salzler *et al.*, 1992).

In 1999, Litherland et al. (1999) found that monocytes from subjects at high risk for IDD aberrantly expressed cyclooxygenase-2 (COX-2, also referred to as prostaglandin synthase-2 or PGS2) and as a result produced increased quantities of prostaglandins. The abnormal expression of COX-2 in pre-IDD subjects contributed to

impaired T cell activation by decreased CD25 expression and interleukin-2 (IL-2) in phytohemagglutin (PHA)-activated T cells when compared to normal controls. Inhibiting COX-2 activity with a specific inhibitor, NS398, significantly increased the CD25 expression and IL-2 production in PHA- activated T cells in pre-IDD subjects (Litherland *et al.*, 1999). Additional studies from the same laboratory report that peritoneal MΦ from NOD mice constitutively express COX-2 and that this expression is responsible for enhanced prostaglandin E2 (PGE2) production (Xie, 1997). Prostaglandins apparently play a role in the pathogenesis of IDD as blocking the cyclooxygenase activity delayed the onset as well, as reduced the incidence of diabetes in the NOD mouse. These data suggest that abnormal expression of COX-2 in humans at high risk for IDD and in the NOD mouse results in increased prostaglandin production by MΦ/monocytes and limits T cell activation by APC. This limitation subsequently may contribute to the pathogenesis of IDD by interference with peripheral tolerance mechanism including generation of regulatory T cells or elimination of autoreactive T cells by activation induced cell death (AICD).

Recent studies suggest that blood monocytes are an immature reservoir of cells with dual potential that can be recruited to the tissues and differentiate into MΦ or DC depending on the tissue microenvironment (Palucka *et al.*, 1998). Peripheral blood monocytes cultured with granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) for six days differentiate into cells with immature DC morphology (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994). These monocyte derived dendritic cells (MDC) express MHC class II molecules as well as low levels of costimulatory molecules CD80 and CD86. They also express CD1a, a tissue DC marker,

lack CD14, a monocyte/MΦ surface receptor, are highly efficient in antigen capture but are poor stimulators of T cells (Sallusto *et al.*, 1995; Sallusto and Lanzavecchia, 1994). After the addition of a maturation stimulus such as tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS) or soluble trimeric CD40L (sCD40L), MDC upregulate MHC class II, CD80, CD86, induces expression of CD83, a DC specific cell surface marker (Zhou and Tedder, 1995; Zhou and Tedder, 1996). Matured DC also decrease mechanisms of antigen capture and become highly immunostimulatory (Sallusto *et al.*, 1995). Kalinski et al. (1997) demonstrated that when high levels of PGE2 are present in culture from inception, MDC do not lose CD14 expression, express lower levels of CD1a and produce significantly lower levels of interleukin-12 (IL-12), a proinflammatory cytokine. Additionally, these MDC exposed to high levels of PGE2 stimulate naïve T cells to produce Th2 cytokines, whereas DC cultured in the absence of high levels of PGE2 stimulated Th1 cytokines (Kalinski *et al.*, 1997). Other studies suggest that exogenous PGE2 added to cultures after monocytes have differentiated into I-MDC acts synergistically with TNF-α to enhance maturation, indicated by increased expression of CD83, and stimulatory capacity of the MDC (Jonuleit *et al.*, 1997; Reddy *et al.*, 1997a). Rieser et al. (1997) also reported that addition of exogenous PGE2 increases IL-12 secretion by MDC by a cAMP mediated mechanism (Rieser *et al.*, 1997) while others have reported that PGE2 down regulates the secretion of IL-12p70 in MDC stimulated with LPS (van der Pouw Kraan *et al.*, 1995; 1996). Collectively, these data suggest that PGE2 has a profound effect on MDC maturation and function but that the effects are highly dependent on the state of MDC maturation. Early exposure of monocytes to PGE2 limits MDC differentiation, for example, maintain CD14 and do not express CD1a,

and promotes Th2 T cell response, while exposure to PGE2 after differentiation into I-MDC results in increased maturation, stimulatory capacity, and secretion of IL-12 (Jonuleit *et al.*, 1997; Kalinski *et al.*, 1997; Rieser *et al.*, 1997).

Prostaglandins mediate their biological action through binding to specific cell surface and nuclear membrane receptors. Four PGE2 receptor subtypes termed EP1, EP2, EP3, and EP4 are coupled to intracellular signaling pathway through GTP binding proteins. EP1 activatrs phosphotidylinositol turnover and increases intracellular CA++ by an unidentified G protein. EP2 and EP4 are coupled to Gs protein and transduce activation of adenylate cyclase resulting in an increase in cAMP (Narumiya *et al.*, 1999). EP3 has multiple isoforms with identical extracellular domains differing only in the cytoplasmic tail which differ in their signal transduction but most are coupled to Gi inhibiting AC and reducing cAMP (Negishi *et al.*, 1995). Secondary messengers, such as cAMP, activated by ligation of prostaglandin receptors control cellular responses by stimulating protein kinases which phosphorylate transcription factors and regulate gene expression.

Recent studies suggest that EP2 and/or EP4 may contribute to the pathogenesis of IDD. Bridgett et al. (1998) describes differential protection in two transgenic lines of NOD mice hyperexpressing a peptide of glutamic acid decarboxylase (GAD), a candidate autoantigen in diabetes. One transgene inserted into the Y chromosome provided no protection from diabetes while the A-line (whose transgne integrated within 6 cM from the centromere of chromosome 15) provided protection from diabetes in the hemizygous state suggesting that protection may be associated with insertional mutagenesis (Bridgett *et al.*, 1998). Candidate genes in the transgene insertion region included EP2 and EP4.

EP2 and EP4 mediate cellular response through increased cAMP and protein kinase A. It is possible that the increased prostaglandins reported in NOD mice mediate their affect through EP2 and/or EP4 and that disruption of one or both of these genes may reduce the level of cAMP generated when prostaglandins are present. Increases in cAMP have been shown to be involved in preventing apoptosis (Boehme and Lenardo, 1993; Critchfield and Lenardo, 1995) and cell cycle progression (Goetzl *et al.*, 1995b; Smit *et al.*, 1998), events that would interrupt peripheral tolerance mechanisms including AICD and possibly lead to an accumulation of autoreactive T cells. Additionally, PGE2 affects the secretion of IL-12 in MDC through cAMP mediated mechanisms (Rieser *et al.*, 1997; van der Pouw Kraan *et al.*, 1995; van der Pouw Kraan *et al.*, 1996) and the maturation of MDC (Jonuleit *et al.*, 1997; Kalinski *et al.*, 1997).

Because prostaglandins affect maturation and function of MDC (cells important in immunity and tolerance) and the expression of COX-2 in monocytes (immature reservoirs of cells with MDC potential) of subjects at risk for IDD, suggest that prostaglandins may contribute to the pathogenesis of IDD by modulation of DC maturation and function. This study was designed to answer the following questions: Chapter 3: Do MDC express COX-1 and COX-2? What is the eicosanoid profile produced by the MDC? Do endogenously produced PG regulate MDC surface antigen expression, capture of antigen, and secretion of IL-12? Chapter 4: Do MDC express PG receptors? What is the number of receptors on the cell surface? Which PG receptors regulate IL-12 production in MDC? Chapter 5: Do MDC generated from subjects at high risk for IDD have similar phenotype and function as MDC generated from normal

controls? Does the aberrant expression of COX-2 and increased PG production in monocytes from subjects at high risk for IDD impairs MDC differentiation?

CHAPTER 2
REVIEW OF LITERATURE

Prostaglandins: Metabolism, Function and Receptors

Eicosanoids

Eicosanoids are a family of oxygenated metabolites of arachidonic acid (AA) that mediate many cellular processes. Over the past 40 years the structures of eicosanoids, which consist of prostaglandins (PG), thromboxane (TBX), hydroxyeicosatetraenoic acids (HETES) and leukotrienes (LT), have been elucidated as well as their cellular location, biosynthesis and action through specific cell surface and nuclear receptors. Liberation of membrane AA by phospholipases (PLA) results from external signals such as hormones, growth factors and cytokines, making AA available for oxygenation by either the linear pathway which results in generation of HETES and LT or by the cyclic pathway which yields PG and TBX (Piomelli, 1993).

Prostaglandin Biosynthesis

The biosynthesis of TBX and PG involves three sequential steps (Figure 2-1). The first step is the release of AA from membrane phospholipid by phospholipase A2 (PLA2). Next, formation of prostaglandin H2 (PGH2) from AA is mediated through two isoenzymes designated cyclooxygenase-1 and –2 (also referred to as COX-1, -2 or prostaglandin synthase-1, -2 or PGS-1, -2). Both isoenzymes catalyze the oxygenation of AA to PGG2 (cyclooxygenase) and reduction to PGH2 (peroxidase) (Smith *et al.*, 1996).

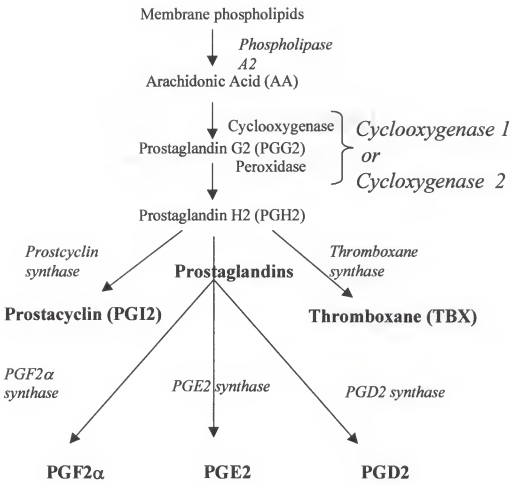


Figure 2-1. Cyclic pathway of arachindonic acid metabolism leading to prostaglandins, prostacyclins and thromboxanes. Enzymes are *italicized.* Cyclooxygenase 1 and 2 (COX-1 and COX-2 also referred to as Prostaglandin Synthase 2, PGS1, and Prostaglandin Synthase 2, PGS2) has both cyclooxygenase and peroxidase activity.

The last step is isomeriztion of PGH2 by specific synthases to produce prostacyclin (PGI2), PG, and TBX, collectively referred to as prostanoids (Figure 1).

The evolutionary pressures that led to two isoforms of COX are not clear. COX-1 is developmentally regulated, constitutively expressed in most tissues, and primarily responsible for cellular homeostasis. In contrast, COX-2 is generally not expressed in unstimulated cells but can be induced to produce large quantities of prostanoids. The induction of COX-2 is not simply a matter of quantity of PG as induction of COX-2 in fibroblast produces little increase in overall PG production (Goetzl *et al.*, 1995a). Additionally, COX-2 is expressed under non-stimulated conditions in the renal cortex and the brain (Seibert *et al.*, 1994). Evidence suggests that the COX-1 and COX-2 occupy different subcellular compartments and may utilize different intracellular pools of AA (Morita *et al.*, 1995; Murakami *et al.*, 1994). Immunostaining reveals that COX-1 is localized to the endoplasmic reticulum (ER) while COX-2 is located in the ER but concentrated in the nuclear envelope (NE). These data suggest that the nuclear location of COX-2 is important in providing PG that interact with nuclear receptors and alter gene expression directly by acting as transcription factors on genes that may be important in cellular growth, replication and differentiation (Murakami *et al.*, 1994; Smith *et al.*, 1996). However, other studies in COX-1 and COX-2 null cells indicate that cells deficient in COX-1 or COX-2 compensate by expression of the alternative COX isoenzyme (Kirtikara *et al.*, 1998).

The human genes for COX-1 and –2 are located on chromosome 9 and 1, respectively. The structural differences in COX-1 and COX-2 genes explain differing patterns of expression of the two enzymes. The COX-2 gene has a TATA box as well as

a number of elements in the 5' promotor region such as a nuclear factor-kb (NFkB) site, CAAT enhancer and a cyclic AMP (cAMP) response element that are generally involved in highly regulated inflammatory genes (Appleby *et al.*, 1994). The COX-1 gene has no TATA, characteristic of a housekeeping gene, and no significant inducible element has been identified in the promotor region. Additionally, mRNA for COX-2 but not COX-1 has long a 3' untranslated region that contains multiple polyadenlyation sites and instability sequences that signal rapid message degradation (Herschman *et al.*, 1997; Smith *et al.*, 1996).

Although COX-1 and –2 have different gene and mRNA structure as well as occupy different locations within the cell, the catalytic characteristics are almost identical except for their susceptibility to inhibition by pharmacological agents. Glucocorticoids inhibit expression of COX-2 while exhibiting little effect on COX-1 (Kujubu and Herschman, 1992; Masferrer *et al.*, 1994). Traditional nonsteroidal anti-inflammatory drugs (NSAID) used to treat inflammatory diseases such as rheumatoid arthritis inhibit both COX-1 and –2; however, a new class of specific inhibitors that target COX-2 (Futaki *et al.*, 1994) has been developed that produce less ulcerogenic and nephrotoxic side effects than NSAID.

Prostaglandin Receptors

Effects of eicosanoids are mediated through binding to specific seven-transmembrane rhodopsin-like G-protein coupled receptors (Figure 2-2). The prostanoid receptors include DP, EP, FP, IP and TP, which bind to PGD2, PGE2, PGF2, PGI2 and TBX, respectively, and transduce secondary intracellular signals by changes in cAMP,

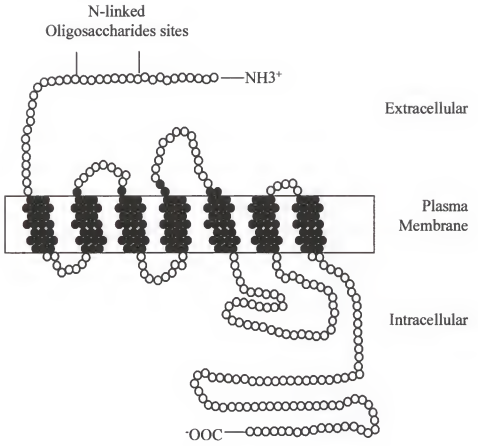


Figure 2-2. Representative structure of prostaglandin receptors which are members of the G-protein coupled rhodopsin-type receptor with 7 putative transmembrane domains. Solid circles indicate hydrophobic amino acids. Conserved motifs include LXAXRXAS/TXNQILDPWVYIL in the seventh transmembrane, GRYXXQXPGS/TWCF in the second extracellular domain, and MXFFGLXXLLXXXAMAXER in the third transmembrane domain are shared by most of the prostanoid receptors. Different isoforms of receptors such as TBX or EP3 have identical amino terminal (ligand binding) and vary only in the carboxy terminal tail (G-protein binding/signal transduction).

Table 2-1: Signal Transduction of prostanoid receptors

Type	Subtype	Isoform	G protein	Second Messanger
DP			Gs	cAMP ↑
EP	EP1		Unidentified	Ca^{++} ↑
	EP2		Gs	cAMP ↑
	EP3		Gi	cAMP ↓
	EP4		Gs	cAMP ↑
FP			Gq	PI response
IP			Gs,Gq	cAMP ↑, PI response
TP		TPα	Gs,Gi	PI response, cAMP ↓
		TPβ	Gq,Gs	PI response, cAMP ↑

phosphotidylinositol (PI) or free CA^{++} concentrations in the cell (Kiriyama *et al.*, 1997)(Table 2-1).

Alignment of the amino acid sequences of the eight prostanoid receptors including the subtypes of EP and TP reveal 28 residues that are conserved. Additionally, all have one or more Asn-X-Ser/Thr in the extracellular amino terminal of the protein that is a consensus sequence for N-glycosylation and is essential for ligand binding (Chiang and Tai, 1998). But despite the conserved sequences, the overall homology of the receptors is only 20-30% even among the subtypes of PGE2 receptors (Narumiya *et al.*, 1999).

Prostaglandins play a role in various central nervous system actions including fever, sleep, acute inflammation and pain, thrombosis, hemostasis, bone resorption, hypertension, and reproduction. However, the potent immunomodulatory affects of PG, particulary PGE2, also suggest a role in immunity and allergy (Goetzl *et al.*, 1995a). Studies in immune cells and PG suggest that the immunomodulatory effects of PGE2 are mediated through increases in cAMP suggesting the involvement of EP2 and/or EP4 (Anastassiou *et al.*, 1992; Bauman *et al.*, 1994; Betz and Fox, 1991; Blaschke *et al.*, 1996; Choung *et al.*, 1998). PGE2-specific effects on immune function will be discussed in subsequent paragraphs.

Dendritic Cells

Initiators of the Immune Response

Adaptive immune response results from antigen uptake, processing and presentation by APC to T cells in lymphoid organs. Among professional APC including DC, MΦ, and B-cells only DC are highly effective at stimulating naïve T cells because of

constitutive expression of costimulatory molecules, CD80 and CD86, and high expression of MHC class II molecules required for T cell activation. Immature DC are distributed throughout the body and equipped with mechanisms to optimize antigen capture including macropinocytosis, receptor-mediated endocytosis vis C-type lectin receptor, Fcγ receptors I and II for uptake of immune complexes or opsonized particles, CD36 and αvβ5 integrins involved in phagocytosis of apotoptic and necrotic cells and entry of intracellular parasites, bacteria, and viruses. Recently, a new receptor, DC-SIGN was identified which, unlike the receptors that mediate antigen uptake which results in antigen processing and presentation of peptide in MHC molecules to T cells, this receptor binds to human immunodeficiency virus (HIV) and transports HIV to the draining lymphnode where the receptor promotes binding and transmission of HIV to T cells (Geijtenbeek *et al.*, 2000). This mechanism, which is important in the pathogenesis of HIV, may be involved in transport of other pathogens to target sites but to date only HIV transport has been reported.

Following capture of antigen, DC migrate to the lymph node via the afferent lymph where they upregulate MHC and costimulatory molecules and activate T cells. Recent data indicate that the microenvironment during antigen capture polarize the DC so that it not only provides signal 1 (peptide bound to MHC) and signal 2 (costimulatory molecules) but also provides signal 3 in the form of release of polarizing cytokines that directs the bias of Th cells towards Th1 or Th2 (Kalinski *et al.*, 1999a). A crucial factor in the polarization towards Th1 or Th2 cytokine production is the presence of IL-12 or IL-4, respectively, during T cell receptor (TCR) engagement (Abbas *et al.*, 1996). DC are known producers of IL-12 but do not produce IL-4 (de Saint-Vis *et al.*, 1998).

Interleukin-12

Interleukin-12 is a heterodimeric cytokine that is involved in priming the naïve T cell for high IFN-γ secretion resulting in a proinflammatory immune response. Heterodimeric IL-12 or IL-12p70 is composed of two subunits of two chains, p35 and p40, that are covalently linked. IL-12p40 is also secreted as a monomer or homodimer generally in excess of 10-100 times that of the biologically active cytokine (Gately, 1999; Sutterwala and Mosser, 1999). The biological activity of IL-12p40 homodimers is not well defined, but several studies in human and mouse suggest that the IL-12p40 homodimer is an IL-12 receptor antagonist (Gillessen *et al.*, 1995; Ling *et al.*, 1995) and may reduce the Th1 response (Yoshimoto *et al.*, 1998). Inducers of IL-12 in DC and other APC include LPS, CpG motifs contained in bacterial DNA, and activated T cells through direct interaction of CD40 with CD40L on the T cell (Macatonia *et al.*, 1995; Shu *et al.*, 1995). Hilkens et al. (1997) found that IFN-γ was an obligatory signal required for DC secretion of the biologically active form of IL-12 via CD40-CD40L (Hilkens *et al.*, 1997). Three well-described inhibitors of IL-12 biosynthesis include IL-10, transforming growth factor-β (TGF-β) and PGE2 (Strassmann *et al.*, 1994; Sutterwala *et al.*, 1997; van der Pouw Kraan *et al.*, 1995).

In vivo and in vitro studies concluded that IL-12 is critical in the development of immunity against intracellular pathogens. Animals treated with anti-IL-12 or that lack the IL-12p40 gene are more susceptible to intracellular pathogen infection (Mattner *et al.*, 1993; Scharton-Kersten *et al.*, 1995). Conversely, overproduction of IL-12 has detrimental consequences including exacerbation of autoimmune disease as reported in

IDD (Trembleau *et al.*, 1995) while administration of IL-12p40 to NOD mice prevented disease (Rothe *et al.*, 1997; Trembleau *et al.*, 1999).

Lineages of Dendritic Cells

The multiple lineages and functions of DC remain complex. Clearly, subsets of DC are derived from hematopoietic progenitor cells. Three distinct hematopoietic DC have been described in the literature (Figure 2-3). Thymic DC and a subset of DC found in the lymph node and spleen originate from a lymphoid lineage. In humans, culturing CD34+ progenitors with IL-3 or flt3-ligand generates lymphoid DC that express CD8a. Shortman et al.(1997) found that in murine lymph nodes two population of DC exist, a CD8a+ (lymphoid DC) and CD8a- (non lymphoid) population. Both populations express similar levels of HLA molecules, and costimulatory molecules CD80 and CD86, but activate T cells differently. T cell proliferation studies demonstrated that lymphoid DC did not activate CD8+ T cells as efficiently as non-lymphoid cells because of an inadequacy of the lymphoid DC to induce IL-2 production. Additionally, lymphoid and non lymphoid DC effectively activated CD4+ T cells but lymphoid DC eliminated the CD4+ T cells after activation by FAS mediated apoptosis. These authors suggest that because of the deletion of T cells via FAS-FASL interaction, lymphoid DC may play a role in peripheral tolerance (Banchereau and Steinman, 1998; Heath *et al.*, 1998; Shortman and Caux, 1997).

A second subset of hematopoietically derived DC can be differentiated by culturing CD34+ precursors with GM-CSF and TNF-α. This yields a DC that is positive for CD1a and expresses markers similar to a Langerhan's cell (LC) or epidermal DC (Caux *et al.*, 1995; Caux *et al.*, 1997). A third hematopoietically derived DC also begins

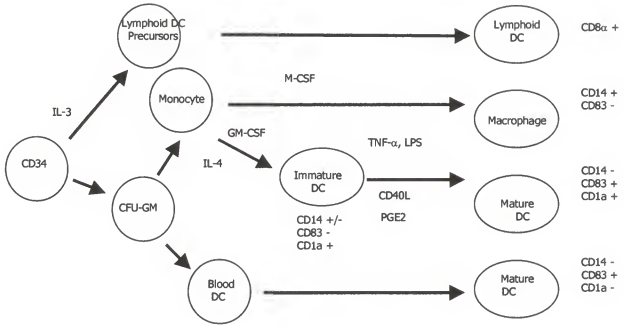


Figure 2-3: Hematopoietic differentiation pathway for myeloid and lymphoid dendritic cells

with a CD34+ progenitor but has a transient CD14 + state. These DC can also be derived from culturing peripheral blood monocytes (CD14+) with GM-CSF and IL-4 for 6-8 days, which yields a monocyte derived dendritic cell (MDC) (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994). The latter two populations are both myeloid lineage DC and express similar levels of CD1a, CD11c, CD40, CD80, CD86, HLA-DR but can be differentiated by expression of CD64 and E-cadherin which are expressed on CD34+ derived DC but not on MDC (Caux *et al.*, 1997; de Saint-Vis *et al.*, 1998).

It has been suggested that the key distinction between lymphoid DC and myeloid DC may be tolerance versus immunity (Banchereau and Steinman, 1998; Shortman *et al.*, 1997); however, recent studies suggest that both lymphoid and myeloid DC have the ability to exhibit tolerogenic activity (Inaba *et al.*, 1998; Suss and Shortman, 1996). Dendritic cells tolerized by TGF-β or IL-10, or genetically engineered DC expressing immunosuppressive activity such as TGF-β, IL-10 or molecules that induce apoptosis such as FASL, may provide mechanisms for tolerance induction for treatment of transplant rejection and autoimmunity (Lu, 1999).

Polarizing Th1 and Th2 Responses in Naïve T cells

Until recently myeloid DC were thought to only stimulated Th1 producing T cells by virtue of their ability to secrete IL-12. Recent studies in vitro and in vivo suggest that myeloid DC can induce Th1 or Th2 cytokine production in naïve T cells (Macatonia *et al.*, 1995; Ronchese *et al.*, 1994; Stumbles *et al.*, 1998). The determining factor in skewing the Th response by the DC is the secretion of IL-12 (Abbas *et al.*, 1996; Snijders *et al.*, 1998) and the amount of IL-12 secreted by the DC is directed by the microenvironmental factors present during antigen capture as well as the antigen itself

(Jonuleit *et al.*, 1997; Kalinski *et al.*, 1999a; Kalinski *et al.*, 1999b). Microenvironmental tissue factors that have been shown to affect IL-12 modulation are PGE2, IL-10, TGFβ and IFN-γ (De Smedt *et al.*, 1997; Kalinski *et al.*, 1998).

Several pathogens, including intracellular bacteria and helminthes, have been shown to modulate secretion of IL-12 by DC (Bancroft *et al.*, 1997; Snijders *et al.*, 1998; Trinchieri, 1997; Trinchieri, 1996). Additionally, other pathogens including intracellular parasites such as Leishmania major induce production of IL-12 inhibitory factors in the host which down modulate IL-12. Viruses such as HIV and measles virus have also been shown to interfere with the production of IL-12 in DC (Karp, 1999; Marshall *et al.*, 1999; Weissman *et al.*, 1996).

Effects of Prostaglandins on Dendritic Cell Maturation and Function

Few studies have examined the effects of PG on the differentiation and maturation of MDC. Kalinski et al. (1997) showed that PGE2 added to cultures at high concentration (10^{-6}M) prevent the differentiating monocyte from acquiring the CD1a marker and losing the monocyte marker, CD14. Additionally, the presence of PGE2 inhibited the ability of the matured MDC to produce IL-12p70 and, therefore, induces Th2 cytokine production (Kalinski *et al.*, 1997). The addition of PGE2 into cultures, in combination with other maturation stimuli, such as TNF-α, LPS, or sCD40L, after the differentiation process from monocyte to I-MDC was complete enhances maturation, migratory, and immunostimulatory capacity of the MDC (Jonuleit *et al.*, 1997; Reddy *et al.*, 1997). Rieser et al. (1997) reported that PGE2 in the absence of LPS stimulated the production of total IL-12 (Rieser *et al.*, 1997) while other have reported that PGE2 is a

potent inhibitor of IL-12 production (Strassmann *et al.*, 1994; Sutterwala *et al.*, 1997; van der Pouw Kraan *et al.*, 1995; van der Pouw Kraan *et al.*, 1996). Although the studies by Reiser et al. (1997) and van der Pouw Kraan et al.(1995) report increased and decreased production of IL-12, respectively, in response to PGE2, both suggest that the mechanism of action is through increase in cAMP production suggesting that the regulation of IL-12 by PGE2 may be through EP2 and/or EP4. Finally, Kalinski et al. (1998) found that fully matured MDC are unresponsive to PGE2 regulation of IL-12. These data suggest that the diverse response by MDC to PGE2 is highly dependent on the maturation stage of the MDC (Kalinski *et al.*, 1998). The autocrine effects of endogenously produced PG on the maturation and function of MDC as well as the distribution of prostaglandin receptors on MDC have not been examined, but are the focus of Chapter 3 and Chapter 4, respectively.

Pathogenesis of Diabetes

Autoimmune insulin dependent diabetes (IDD) results from a cell mediated immune response that destroys the insulin producing cells of the pancreas (β cells of the islets of Langerhan's). Although T cells are critical to the pathogenesis of IDD, MΦ and DC may contribute as the initiation of the autoimmune process begins with the presentation of β cell specific antigens by APC to autoreactive CD4+ T cells. Alternatively, DC have been shown to be important in induction of tolerance through generation of regulatory T cells (Clare-Salzler *et al.*, 1992), anergy (Kirk *et al.*, 1997; Larsen *et al.*, 1996), and AICD (Suss and Shortman, 1996). Initially, MΦ and DC infiltrate the islet of Langerhan's (Dahlen *et al.*, 1998) followed by T cell infiltration and Th1 mediated destruction of the insulin secreting β cells. The secretion of IL-12 by the

APC polarizes the immune response toward Th1 and induces the secretion of interferon-γ (IFN-γ) by Th1 cells. IFN-γ activates MΦ causing release of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) as well as free radicals. This release of cytokines results in increased apoptosis and/or necrosis of β cells (Dahlen *et al.*, 1998; Delaney *et al.*, 1997; Heitmeier *et al.*, 1997).

IDD susceptibility is associated with many genes including genes of MHC class II. The most significant association is with MHC class II haplotypes DR3-DQB1*201 and DR4-DQB1*302. Although the MHC molecules are critical for presentation of antigen to CD4+ T cells and the pathogenesis of IDD attributed to accumulation of islet antigen specific Th1 cells, the mechanism of immune dysregulation in IDD has not been fully defined. The accumulation of autoreactive T cells may result from failure of elimination by activation induced cell death, induction of anergy, or deficient generation of regulatory cells caused by impaired antigen presentation. In humans with IDD as well as the murine model, the NOD mouse, inability of the APC to activate T cells has been described including decreased clustering, decreased production of IL-2, and deficient activation of regulatory cells (Clare-Salzler and Mullen, 1992; Jansen *et al.*, 1995).

Prostaglandins and Pathogenesis of IDD in Humans and NOD

As stated previously, PG, especially PGE2, have diverse effects on the immune response. PGE2 can modulate T cell differentiation, tissue migration and effector function. PGE2 protects thymocytes from activation-induced apoptosis through thymocyte expression of EP2 and increased cAMP (Goetzl *et al.*, 1995b). Migration of T cells across basement membranes is enhanced by PGE2 (through EP2 and increases in cAMP) and secretion of matrix metallproteinases (Leppert *et al.*, 1995). Finally, PGE2

modulates T cell effector function by inhibiting the production of IL-2 and IFN-γ and the expression of CD25 (α-chain, IL-2 high affinity receptor) (Anastassiou *et al.*, 1992; Hancock *et al.*, 1988; Katamura *et al.*, 1995). The inhibition of CD25 and IL-2 production leads to inhibition of proliferation and subsequently blocked AICD that is required for elimination of T cells after activation (Bauman *et al.*, 1994). One group reports that previous studies on the effects of PGE2 used high concentrations that are above physiologic levels, and that physiologically relevant concentrations of PGE2 actually enhance the production of IFN-γ by antigen-stimulated Th1 cells (Bloom *et al.*, 1999). A recent report suggests that different strains of mice have sensitivity differences to the suppressive effect of PGE2 possibly through the number of prostaglandin receptors, and that this difference may account for preferential polarization to a Th2 response in Balb/c mice (Kuroda *et al.*, 2000). Studies in the NOD strain have not been reported.

Until recently, the suppressive effects of PGE2 in NOD mice had not been studied; however, Ganapathy et al. (2000) recently suggested that PGE2 may be a less effective negative regulator of activated CD8+ T cells IFN-γ secretion in NOD than in Balb/c mice (Ganapathy *et al.*, 2000). PG apparently play a role in the pathogenesis of disease since blocking endogenous production of PG delays onset and decreases incidence of diabetes in NOD mice (Xie, 1997). Xie (1997) reported that peritoneal MΦ from NOD mice constitutively expressed the normally inducible COX-2 enzyme and that this constitutive expression was responsible for increased production of PGE2. Litherland et al. (1999) reported similar findings, aberrant expression of COX-2 in human

peripheral blood monocytes, from subjects at increased risk for IDD (Litherland *et al.*, 1999).

The possible role of PG receptors in the pathogenesis of diabetes was suggested by Bridgett et al.(1998) when two transgenic lines of NOD mice overexpressing the putative autoantigen GAD (Kaufman *et al.*, 1993) in pancreatic β cells had differential protection from diabetes. The Y-line integrated in the Y chromosome but showed similar incidence of diabetes in male NOD than the standard NOD males, while the A-line which incorporated into the proximal end of chromosome 15, where the genes for murine EP2 and EP4 are located, exhibited a markedly lower incidence of diabetes in both sexes. Additionally, the ratio of IFN-γ to IL-10 transcripts was reduced in the A-line suggesting that the insertional mutagenesis is a possible mechanism in the A line protection from diabetes (Bridgett *et al.*, 1998).

Collectively, these studies suggest that PG could play a major role in the pathogenesis of diabetes. First, activation of regulatory T cells for induction of tolerance requires a highly immunostimulatory APC such as MDC. Subjects with a high risk for IDD and NOD mice aberrantly express COX-2 and produce PG that could affect differentiation and the immunostimulatory capacity of MDC. Second, increased prostaglandin production during T cell activation may lead to decreased IL-2 production and increased cAMP mediated antiapoptotic that could impair activation induced cell death, a mechanism required for peripheral tolerance. Chapter 5 describes a comparative study of MDC in normal human controls and subjects at high risk for IDD to ascertain if atypical expression of COX-2 in subjects at high risk for IDD impair MDC maturation and function.

CHAPTER 3
AUTOREGULATION OF HUMAN MONOCYTE DERIVED DENDRITIC CELL MATURATION AND FUNCTION BY CYCLOOXYGENASE-2 MEDIATED PROSTAGLANDIN PRODUCTION

Review of Literature

Prostaglandins are important lipid mediators for a wide variety of physiological cellular functions (Crofford, 1997; Morita *et al.*, 1995; Smith *et al.*, 1996; Williams and Shacter, 1997). Prostaglandin synthesis is regulated by a series of steps involving the release of endogenous arachidonic acid (AA) by phospholipase A2 (PLA_2), and the subsequent conversion of AA to prostaglandin H2 (PGH2). Conversion of AA to PGH2, the first and rate limiting step in prostaglandin biosynthesis, is mediated through two isoenzymes, cyclooxygenase 1 (COX-1 also referred to as prostaglandin synthase-1, PGS-1) and cyclooxygenase-2 (COX-2, also known as PGS-2). Constitutively expressed COX-1 is primarily responsible for cellular homeostasis while COX-2 is inducible and is responsible for high-level production of prostanoids that modulate inflammation and mitogensis (Brock *et al.*, 1996). Monocyte expression of COX-2 is induced by a variety of stimuli including LPS, PMA, and IL-1β (Hwang *et al.*, 1997; Yamaoka *et al.*, 1998). In monocytes, LPS upregulates COX-2 through induction of GM-CSF while IL-1β enhances and stabilizes COX-2 transcripts. In several cell types including monocytes, COX-2 expression is suppressd by IL-4, IL-10 and IL-13 via transcriptional and postranscriptional regulation (Endo *et al.*, 1996).

Expression of COX enzymes, prostanoid production, and the autocrine effects of these molecules have not been reported for MDC. Previous studies, however, described the effects of exogenous prostaglandins on MDC maturation and function. Kalinsky et al. (1997) demonstrated that high concentration (10^{-6} M) of exogenous PGE2 added to monocytes in the presence of GM-CSF and IL-4 profoundly modulated MDC development as these cells do not lose CD14, expressed low levels of CD1a, and produced significantly less IL-12p70 and higher levels of IL-10 (Kalinski *et al.*, 1997). Additionally, MDC derived under these conditions stimulated Th2 responses whereas MDC cultured without exogenous PGE2 stimulated Th1 responses. Other studies demonstrated that exogenous PGE2 (10^{-6}M), when added to cultures following monocyte differentiation into I-MDC, synergized with TNF-α or TNF-α/IL-1/IL-6 at 10^{-8}M to induce maturation, immuno-stimulatory capacity and IL-12 production (Jonuleit *et al.*, 1997; Reddy *et al.*, 1997a). These published studies demonstrate that exogenous prostanoids markedly affect MDC maturation and function and that the effect is highly dependent on the developmental stage of the MDC. Preliminary data from our laboratory suggested that MDC express COX-2 constitutively; therefore, we asked if MDC expressed COX-1 and COX-2 and produced prostaglandins that in an autocrine manner regulated MDC maturation and function?

Materials and Methods

Isolation of Monocytes and Dendritic Cell Culture Conditions

PBMC were isolated from buffy coats from one unit of whole human blood using Histopaque Ficoll (Sigma, 1.077, endotoxin tested, St. Louis, MO). Cells were washed two times with Dulbecco's PBS (DPBS), Ca^{++} and Mg^{++} free (Cellgro, endotoxin tested)

and resuspended in RPMI 1640 media with L-glutamine (Gibco, BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, endotoxin tested, Logan, UT), and 1% streptomycin, penicillin, neomycin (Sigma). PBMC were allowed to adhere for 2 hours at 37°C, 5% CO_2, 100% humidity and non-adherent cells were washed away with DPBS. Complete RPMI tested negative for endotoxin (<2.0 EU/ml) (E-Toxate® Kit, Sigma). Adherent cells were cultured for 6 days in complete RPMI supplement with 500 U (50 ng/ml) GM-CSF (Endogen, Woburn, MA) and 500-1000 U IL-4 (R&D Systems, Minneapolis, MN) to generate I-MDC (Sallusto and Lanzavecchia, 1994). To generate M-MDC, day 6 I-MDC were harvested, washed and re-plated at 3.0×10^5 cells/ml and supplemented with 1 μg/ml sCD40L (gift from Immunex, Seattle WA) and/or 1000 U of IFN-γ (human recombinant, Endogen). Some cultures were supplemented with NS-398 (Cayman Chemical, Cambridge, MA), a specific COX-2 inhibitor (1μg/ml), or Indomethacin (Sigma) (10μg/ml), a COX-1 and COX-2 inhibitor.

Surface and Internal Protein Analysis

The following monoclonal antibodies directed against surface or internal proteins were used: CD14, HLA-DR (Becton-Dickinson, San Jose, CA), CD1a, CD86, CD80, CD40 (Pharmingen, San Diego, CA), CD83 (Coulter-Immunotech, Miami, FL), and COX-2 (FITC, Cayman Chemical). Appropriate fluorochrome labeled isotype control antibodies were used. Cells were suspended in PBS with 1% BSA (reagent grade, Sigma) and 0.1% Sodium Azide (Sigma). For surface marker labeling, cells were incubated with 1 μg of fluorochrome conjugated antibody/1×10^6 cells for 20 minutes at room temperature, then washed one time with 2.0 ml of PBS and resuspended in 500 μl of 1% formaldehyde in PBS. Intracellular labeling of COX-2 was performed as

previously described (Litherland *et al.*, 1999). All cells were analyzed on Becton-Dickinson FACSCalibur or FACSort. Flow Cytometry data were analyzed and median fluorescent intensity calculated with WinMidi© (Version 2.7, Joseph Trotter).

Cultured MDC were washed with PBS supplemented with protease inhibitors (1 μg/ml of each leupeptin, pepstatin and aprotinin, Sigma) and 5 μg/ml indomethacin and frozen at –70°C. Lysates were thawed, sonicated and centrifuged for 10 minutes at 14,000 rpm. Equal quantities of protein were separated by SDS-PAGE with a 10% Tris-HCL gel (Biorad), and transferred to nitrocellulose (Optitran, Schleicher and Schull, Keene, NH.) Nitrocellose was probe with monoclonal antibodies directed against COX-1 and COX-2 (Cayman Chemical) and secondary antibodies (anti-mouse IgG-horseradish peroxidase, Amersham, Arlington Heights, IL). Peroxidase activity was detected by chemiluminescence (ECL Western Blotting detection system, Amersham Life Sciences).

PGE2 and Cytokine Assays

Supernatants from cultures of MDC were harvested for analysis of PGE2 and IL-12. I-MDC were cultured for 6 days, washed from the plate, counted and re-plated at $3X10^5$ cells/ml in media containing GM-CSF and IL-4. I-MDC were cultured for an additional 48 hours before supernatants were harvested for analysis. Supernatants from maturing M-MDC were prepared by harvesting I-MDC on day 6, re-plating these cells at the same density in media containing GM-CSF, IL-4 and maturation stimuli. Cells were cultured for an additional 48 hours and then supernatants harvested. MDC culture supernatants from various conditions were analyzed for IL-12p70 and IL-12p40 (gift from Dr. Maurice Gately, Hoffman Roche, Nutely, NJ), by ELISA in duplicate as previously described (Zhang *et al.*, 1994). The lower limit of IL-12p40 and IL-12p70

detection in this assay is 15.6 pg/ml. Supernatants for IL-10 were measured by ELISA (Endogen, capture antibody clone 9D7 and detection antibody, clone 12G8 biotinylated). The lower limit of detection for IL-10 is 20.5 pg/ml. PGE2, PGD2, PGF2a and thrombaxane were measured by competitive enzyme immunoassay (Cayman Chemical). The limit of detection for this assay is 30 pg/ml. Cytokine and prostanoid values were standardized to pg/ml/1 X 10^6 cells.

Antigen Uptake Measured by FITC-Dextran and Lucifer Yellow

Mannose receptor-mediated endocytosis was measured by the cellular uptake of FITC-Dextran (FD, 40,000 MW, Molecular Probes, Eugene, OR) and quantitated by flow cytometry. Approximately 1.5 X 10^5 MDC were incubated in complete RPMI with 25 mM Hepes and 1 mg/ml of FITC-Detran for 1 hour at 0 °C and 37°C. After 1 hour, cells were washed four times with ice-cold 1X PBS with 0.1% sodium azide and immediately run on the flow cytometer. Fluid-phase endocytosis by macropinocytosis was measured by cellular uptake exactly as described for uptake of FD except 1 mg/ml Lucifer Yellow (LY, dipotassium salt, Molecular Probes) was used. Mean fluorescent intensity (MFI) of 37°C – 0°C (baseline) was used to evaluate antigen uptake in different maturation states of MDC.

Results

MDC Express COX-1 and COX-2

To determine whether MDC express COX-2, we employed an established protocol employing GM-CSF and IL-4 to generate I-MDC from peripheral blood monocytes (Sallusto and Lanzavecchia, 1994). After six days in culture, I-MDC were harvested and washed then re-plated and cultured for an additional 48 hours in new media

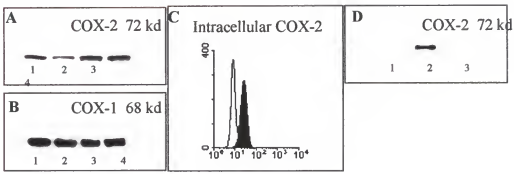


Figure 3-1. Monocyte derived DC express COX-2. SDS page electrophoresis with a 10% Tris-HCL gel loaded with 30 μg of protein from MDC cell lysates and 10 μg of protein from monocytes cell lysates. (A) COX-2 and (B) COX-1 expression in Lane 1: I-MDC (GM-CSF and IL-4 for 8 days), Lane 2: M-MDC matured with 1μg/ml CD40L only , Lane 3: M-MDC matured with 1 μg/ml CD40L and 1000 U/ml IFN-γ. (C) Intracellular staining of COX-2 with FITC conjugated monoclonal antibody (filled histogram is anti-COX-2 and open histogram is isotype control) in I- MDC (A) (M-MDC not shown.) . (D) COX-2 expression in monocytes cultured for 24 hours Lane 1:complete RPMI, Lane 2: 1 μg/ml LPS, Lane 3: 1 μg/ml LPS with 500 U/ml of IL-4.

containing GM-CSF and IL-4. MDC maturation was stimulated by culturing I-MDC with either soluble trimeric CD40L (sCD40L) in the presence or absence of human recombinant IFN-γ for the same 48-hour period. Cells and culture supernatants were harvested at the 48 hours time point for analysis.

We first analyzed the MDC from these cultures for COX-1 and COX-2 expression by intracellular flow cytometry (Litherland *et al.*, 1999) and immunoblotting. As seen in Figure 1A and 1B, I-MDC, I-MDC stimulated with IFN-γ only, sCD40L only, and sCD40L/IFN-γ constitutively express COX-1 and COX-2. We were also able to detect intracellular COX-2 expression by flow cytometry (see Figure 3-1C) and further establish expression in MDC. This is in marked contrast to monocytes that express COX-1 constitutively (data not shown) but require LPS induction for COX-2 expression (Figure 1D). Of interest, while monocyte COX-2 is readily suppressed by 500 U/ml of IL-4 (Figure 3-1D, Lane 3), the same concentration of IL-4 present in MDC cultures does not regulate COX-2 in either I- or M-MDC (Figure 3-1A and B). Interleukin-10, also, does not suppress COX-2 (data not shown). These findings with MDC are in marked contrast to several studies demonstrating that LPS-induced monocyte COX-2 expression is readily down regulated by anti-inflammatory cytokines IL-4, IL-10 and IL-13 (Endo *et al.*, 1996; Niiro *et al.*, 1995). However, our results are similar to findings by Maloney et al. (1998) that showed COX-2 induced by LPS or GM-CSF in neutrophils was not down regulated by IL-4 or IL-10 (Maloney *et al.*, 1998). These data suggest that GM-CSF, IL-4 or factors produced in culture by monocytes or the differentiating process induce COX-2 in manner that provides resistance to cytokine regulation.

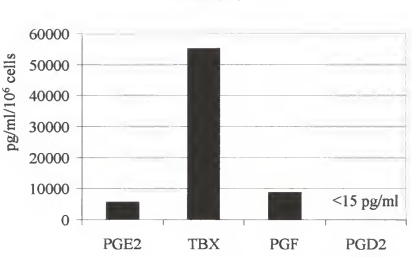


Figure 3-2. Prostanoid production in immature monocyte derived dendritic cells. TBX = Thromoboxane, PGF = PGF2α. Concentration of agonist/antagonist is1 X 10^{-6} M. Data represents one of at least three sets performed. Prostanoids are expressed in pg/ml/ 1 X 10^6 cells.

Prostanoid Production by I-MDC and M-MDC

Next, COX-1 and COX-2 mediated prostanoid production by MDC populations was examined. Supernatants of I- and maturing M-MDC cultured in the presence and absence of NS-398, a specific COX-2 inhibitor, or indomethacin, a COX-1 and –2 inhibitor, added during the last 48 hours of cell culture were analyzed. Results show MDC (in the absence of inhibitors) spontaneously produce thromboxane (TBX) > PGE2 > prostacyclin but no PGD2 (Figure 3-2). NS-398 and indomethacin significantly reduce PGE2 production to a similar degree, suggesting that prostanoid synthesis occurs predominantly through COX-2 in I-MDC (Figure 3-3). It is possible that small numbers of residual monocytes, approximately 1% of our cultures, produce large quantities of prostanoids and account for COX-2-mediated prostaglandins. Although this possibility exists, monocytes do not express COX-2 during culture without activation. Furthermore, the expression of this enzyme is readily suppressed in monocytes by the presence of IL-4 in the culture (see Figure 3-1d).

Next, production of prostaglandin by I-MDC undergoing maturation when stimulated for 48 hours with sCD40L was evaluated. MDC cultured in these conditions synthesize two-fold more PGE2 but utilize primarily COX-1 since indomethacin but not NS-398 markedly reduced prostaglandin production (Figure 3). We also evaluated the effects of IFN-γ on sCD40L mediated maturation because this cytokine in combination with sCD40L strongly influences MDC function and development especially secretion of IL-12p70 (Hilkens *et al.*, 1997). When MDC are matured with sCD40L in combination with IFN-γ, a 3-4 fold increase in COX-2 mediated PGE2 prostaglandin production occurs which is reduced toI-MDC levels in the presence of NS-398 (Figure 3-2).

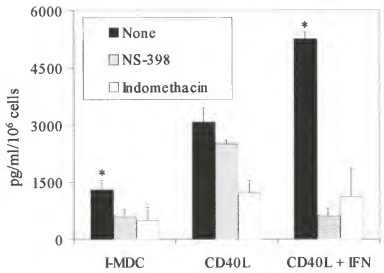


Figure 3-3. PGE2 production by I- and M-MDC is suppressed by a COX-2 specific inhibitor. PGE2 was measured by competitive immunoassay and expressed as pg/ml/1 X 10^6 cells. Data represents the mean and SEM of at least four independent experiments, *$p \leq 0.05$ as calculated by one-way ANOVA.

Interferon-γ also stimulated a two-fold increase in COX-2 dependent PGE2 production from I-MDC. The effects of IFN-γ on COX-2-mediated prostaglandin production by in I-MDC and maturing MDC may be related to the increased access of COX-2 to substrate, as this cytokine readily stimulates AA release through G-protein mediated activation of PLA2 (Visnjic *et al.*, 1997). Overall, these data suggest that the synthesis of prostanoids through COX-2 is the default pathway for I-MDC, whereas stimulation of I-MDC by sCD40L in the absence of IFN-γ switches AA metabolism to COX-1. However, when inflammatory stimuli such as IFN-γ or LPS and TNF-α are present, COX-2-mediated prostaglandin synthesis again predominates.

The quantity of PGE2 produced by MDC (10^{-9}M) is relatively small in comparison to LPS activated monocytes which produce micromolar quantities of PGE2. It is not readily evident why quantitative differences in prostaglandin metabolism exist between these two types of myeloid cells. Based on the Western blots, monocytes do not express a greater mass of COX-2 than MDC. Therefore, it may be that the presence of IL-4 in MDC cultures limits PLA2 activity and substrate availability (Nassar *et al.*, 1994). However, culturing MDC in the absence of IL-4 for 24 hours increased PGE2 production, but the prostaglandin levels remained in the nanomolar range. Alternatively, higher levels of AA may be liberated when monocytes are stimulated by LPS. However, stimulation of maturing MDC with LPS or TNF-a leads to only nanomolar quantities of PGE2. Thus the quantitative set point for production of prostanoids by MDC appears to be substantially lower than that of macrophages or monocytes.

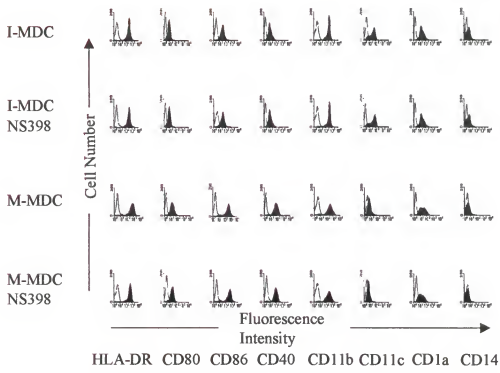


Figure 3-4. Blocking cyclooxygenase activity does not affect expression of HLA-DR and costimulatory molecules on I-MDC or MDC. I-MDC were cultured with GM-CSF and IL-4 in presence and absence of COX-2 inhibitor, NS-398. M-MDC cultured for six days with GM-CSF and IL-4 then matured in the presence or absence of NS-398 with soluble trimeric CD40L and IFN-γ. MDC were stained with antibodies to cell surface markers conjugated to fluorochromes listed in Materials and Methods. Filled histogram indicates cell surface staining and open histogram represents isotype antibody staining.

COX-2 Mediated Prostaglandin Synthesis Promotes MDC Maturation

To establish whether endogenous prostaglandins affect differentiation of I-MDC from monocytes, surface antigen expression of CD1a, CD14, CD40, CD80, CD86, CD83 and HLA-DR on these cells cultured in the presence and absence of NS-398 was analyzed (Figure 3-4). These data demonstrate that blocking endogenous COX-2 mediated prostanoid production did not affect expression of CD1a, HLA-DR or the expression of the co-stimulatory molecules during differentiation from monocytes to I-MDC (Figure 3-4). These data are consistent with Kalinski et al. (1997) who reported that MDC exposed to 10^{-9} M exogenous PGE2, equivalent to levels produced by I-MDC, did not affect MDC differentiation from monocytes (Kalinski *et al.*, 1997).

The same series of antigen markers on I-MDC matured with sCD40L and sCD40L with IFN-γ in the presence of either NS-398 or indomethacin were examined. Again, blocking COX-2 in MDC stimulated with sCD40L/IFN-γ did not modify expression of CD40, CD80, CD86 or HLA-DR (Figure 3-4). Additionally, when indomethacin was used to block COX-1, the predominant enzyme metabolizing arachidonate in MDC matured with sCD40L alone, likewise there was no affect on expression of these same antigens. Previous studies demonstrated that micromolar concentrations of PGE2 enhanced I-MDC maturation when used in cell culture in combination with LPS, TNF-α, or a mixture of inflammatory cytokines (Jonuleit *et al.*, 1997; Reddy *et al.*, 1997a). However, in the present studies we did not find that reducing prostaglandins limited MDC maturation based on the expression of these antigens. It appears that large quantities of PGE2, such as that produced by macrophages, are required to modulate surface molecules such as CD86.

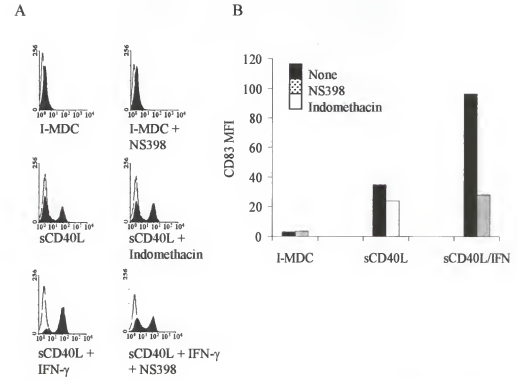


Figure 3-5. Endogenous prostaglandins regulate CD83 in M-MDC matured with sCD40L and IFN-γ. (A) Flow cytometric analysis of CD83 in I-MDC and M-MDC matured with sCD40L alone with our without Indomethacin, and with sCD40L/ IFN-γ in the presence or absence of NS398. (B) Bar graph displays comparison of the median fluorescence intensity (MFI) in I-MDC and M-MDC matured with sCD40L alone and sCD40L/ IFN-γ in the presence or absence of NS398 or Indomethacin. These results are representative of four independent experiments.

Prostanoids produced by MDC are, however, not without affect on MDC maturation. Blocking COX-2 with NS-398 profoundly inhibited CD83 expression following sCD40L/IFN-γ stimulation (Figure 3-5). The predominant effect of prostanoids appears to be mediated through COX-2, as the addition of indomethacin did not enhance this effect (data not shown). When MDC were stimulated with sCD40L alone, substantially lower levels of CD83 expression were achieved. Since MDC stimulated in this manner produce PGE2 primarily through COX-1 we blocked prostaglandin production with indomethacin and evaluated CD83 expression. Unlike I-MDC matured with sCD40L and IFN-γ, the COX inhibitor did little to affect CD83 expression in these conditions. These data are consistent with the previous reports suggesting that PGE2 increases CD83 expression on MDC. However, these studies employed micromolar concentrations of PGE2 to enhance CD83 expression (Jonuleit *et al.*, 1997). Although lower doses of PGE2 equivalent to that made by MDC were not tested in these reports, it may be that sCD40L provides a qualitatively different stimulus than LPS, TNF-α or a combination of inflammatory cytokines such that nanomolar levels of prostaglandins are effective. Based on the present findings, it appears that lower levels of endogenous prostaglandins uniquely stimulate expression of CD83 in contrast to other maturation antigens, e.g. CD86.

Endogenous Prostanoid Production Affects Secretion of IL-12

MDC secretion of the Th1 polarizing cytokine, IL-12, has been extensively studied (Cella *et al.*, 1996; Hilkens *et al.*, 1996; Hilkens *et al.*, 1997; Rieser *et al.*, 1997; Snijders *et al.*, 1996; Snijders *et al.*, 1998). To examine the effect of endogenous

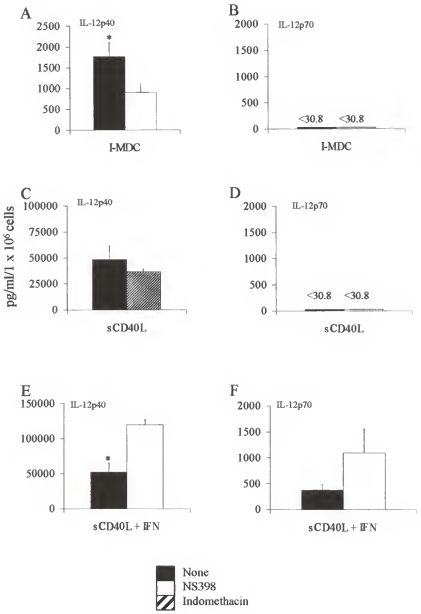


Figure 3-6. Endogenous prostaglandins autoregulate IL-12p40 and IL-12p70 production by I-MDC and M-MDC. Supernatants from I-MDC and M-MDC in the presence or absence of NS-398 or Indomethacin were analyzed for IL-12p40, IL-12p70 by ELISA. (A) IL-12p40 in I-MDC, *p=0.045, (B) IL-12p70 in I-MDC, (C) IL-12p40 in MDC undergoing maturation with sCD40L, (D) IL-12p70 in MDC undergoing maturation with sCD40L (E) IL-12p40 in sCD40L/IFN-γ matured MDC, *p =0.007 (F) IL-12p70 in sCD40L/IFN-γ matured MDC, p=0.06. Conditions analyzed by paired t test.

prostaglandins on secretion of IL-12p40 and IL-12p70, MDC were prepared and assayed for both forms of this cytokine in the supernatants in the presence and absence of COX inhibitors. We chose to study IL-12 production during maturation of MDC using sCD40L alone, and in combination with IFN-γ, the latter combination stimulating production of biologically active IL-12p70 (Hilkens *et al.*, 1997). Consistent with previous reports, we found that I-MDC produced only IL-12p40 and did not produce IL-12p70 (Cella *et al.*, 1996; Snijders *et al.*, 1996). When I-MDC were cultured in the presence of NS-398 for 48 hours IL-12p40 levels were significantly reduced (Figure 3-6). The inhibition of IL-12 by indomethacin was not different from that of NS-398, suggesting the effects of prostanoids on this cytokine are predominantly mediated by the COX-2 isoform. These results are consistent with those of Rieser et al. (1997) who showed an increase in total IL-12 when I-MDC are exposed to PGE2 or other compounds which increase intracellular cAMP (Rieser *et al.*, 1997). In marked contrast, I-MDC undergoing maturation for 48 hours with sCD40L and IFN–γ in the presence of COX-2 inhibitor, significantly increased IL-12p40 production ($p=0.007$) and increased, but not significantly, IL-12p70 production ($p=0.068$)(Figure 3-6). These findings mirror previous studies that showed that the addition of PGE2 to cell culture suppressed IL-12p70 production by maturing MDC (Snijders *et al.*, 1996; van der Pouw Kraan *et al.*, 1995). Studies show PGE2 to be the predominant prostanoid suppressing IL-12 production. Prostacyclin had similar but lesser effects than PGE2 on secretion of IL-12 while TBX and metabolites PGD2 had little to no effect (Figure 3-7). Collectively, these data further demonstrate that prostanoids produced via COX-2 modulate MDC function

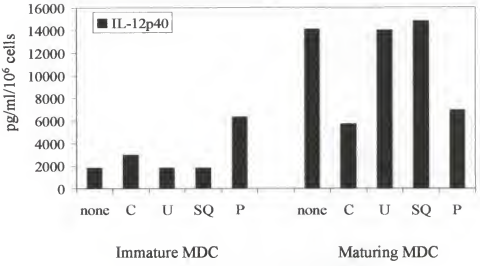


Figure 3-7. Agonist/antagonist stimulation and IL-12 production in immature and maturing MDC. C=carbacyclin, prostacyclin agonist; U=U46619, thromboxane agonist; SQ=SQ29548, thromboxane antagonist; P=PGE2. Concentration of agonist/antagonist is 1 X 10^{-6} M. IL-12 is expressed in pg/ml/1 X 10^{6} cells.

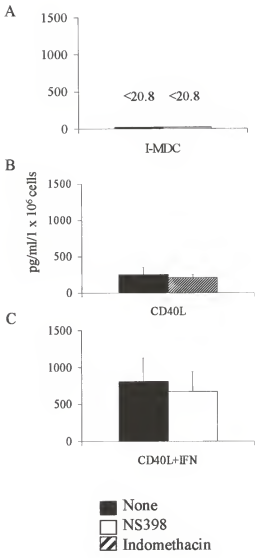


Figure 3-8. Endogenous prostaglandins do not significantly affect IL-10 production in MDC. IL-10 production measure by direct ELISA in the presence or absence of COX inhibitors (A) I-MDC presence and absence of NS398, (B) sCD40L matured MDC with and without Indomethacin, (C) sCD40L/IFN-γ matured MDC with and without NS398. No statistically differences noted using paired t test.

and markedly affect the secretion of IL-12. However, the effect is dependent on the state of differentiation of these cells.

IL-10 Production by MDC is Not Regulated by Prostaglandin Synthesis

Previous studies in murine macrophages demonstrated that IL-10 production in LPS stimulated macrophages occurred through a cAMP/PGE2 dependent mechanism (Strassmann *et al.*, 1994). Therefore, the production of IL-10 in I-MDC and maturing MDC was evaluated. I-MDC do not produce detectable levels of IL-10 while M-MDC matured with soluble sCD40L alone or with and IFN-γ produce low levels that are not significantly reduced with NS-398 or Indomethacin (Figure 3-8). These experiments do not suggest that prostaglandins produced by MDC stimulate IL-10 production. Furthermore, they demonstrate that prostaglandin mediated suppression of IL-12 in maturing MDC is mediated directly by endogenous PGE2 and not through its effect on IL-10.

Prostaglandins Do Not Significantly Affect Antigen Uptake

Finally, the ability of the maturing MDC to shut down antigen uptake in the presence and absence of endogenously produced prostaglandins as well as exogenously added PGE2 was measured by the MDC ability to uptake two fluorescent dyes. I-MDC express potent ability to uptake external molecules by two main mechanism, receptor-mediated endocytosis and macropinocytosis. Two fluorescent markers, FITC-Dextran (FD) and Lucifer Yellow (LY) measure receptor-mediated endocytosis and macropinocytosis, respectively. MDC that are mature decrease the ability to take up these markers thus reducing their antigen uptake. Figure 3-9 shows the reduce capacity

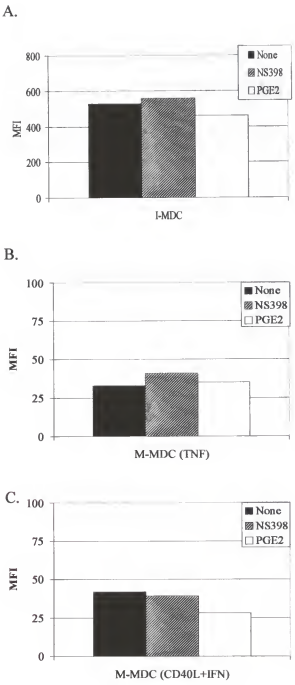


Figure 3-9: Endogenous and exogenous prostaglandins do not significantly affect antigen uptake in MDC. (A) I-MDC (B) TNF-α matured MDC (C) sCD40L and IFN-γ matured MDC incubated with 1 μg/ml of FITC dextran at 37oC for 1 hour in the presence or absence of NS398 or PGE2. MFI represents median fluorescence intensity. Similar results obtained with Lucifer yellow (data not shown).

of M-MDC to take up FD (the higher the MFI the higher the antigen uptake) and that endogenously produced prostaglandins as well as exogenously added PGE2 has no affect on the maturing MDC to shut down the antigen uptake machinery.

Discussion

This is the first report demonstrating that I-MDC and M-MDC constitutively express both cyclooxygenases, COX-1, and the normally inducible COX-2, and synthesize nanomolar quantities of PGE2. The predominant isoform of COX utilized to produce prostanoids by I-MDC is COX-2. An interesting finding of this study was that when I-MDC undergo maturation with sCD40L alone, PGE2 synthesis proceeds through COX-1. In contrast, MDC stimulated with sCD40L in combination with IFN-γ leads to higher levels of PGE2, but production reverts back to the COX-2 pathway. The observation that prostaglandin synthesis fluctuates from one COX isoform to the other is not a novel finding. Previous studies demonstrated that this phenomena occurs as a consequence of the coupling of COX isoforms to distinct PLA2 isoenzymes, e.g. cytoplasmic PLA2 to COX-2, and linkage of apparently discrete pools of AA to either COX-1 or COX-2 (Reddy *et al*., 1997b). Supporting these published studies, mouse macrophages expressing both COX-1 and COX-2 produce PGE2 only through COX-1 when AA added to cultures, whereas IFN-γ stimulates only COX-2 mediated prostaglandin synthesis.

The regulation of COX-2 expression in MDC is unlike that of the precursor monocyte population as this cell is highly resistant to suppression by the anti-inflammatory cytokine IL-4 and IL-10. The reason for the marked alteration in COX-2

regulation is not apparent but may be related to the continuous presence of GM-CSF *in vitro*. Another possibility is that long-term culture or long-term exposure to IL-4 may also diminish the MDC response to this cytokine. Alternatively, studies have demonstrated that freshly isolated monocytes were more responsive to IL-4 induced TNF-α suppression than macrophages cultured for 7 days (Hart *et al.*, 1995). These studies conclude that monocyte responses to immunoregulatory cytokines such as IL-4 may not mirror responses by their differentiated or activated counterparts (Hart *et al.*, 1995). This suggestion is also supported by the studies of Maloney et al. (1998) in which neutrophil expression of COX-2 was likewise found resistant to IL-4, IL-10 and Il-13 (Maloney *et al.*, 1998). Therefore, the pathway of myeloid differentiation or maturation may dictate the responsiveness of COX-2 to anti-inflammatory cytokines.

The production of prostaglandins appears to autoregulate some aspects of MDC maturation, e.g. CD83, and function, e.g. IL-12 production by MDC. These finding show that endogenous prostanoids generated through COX-2 *in vitro* do not interfere with the expression of HLA-DR and co-stimulatory molecules on I-MDC. This result was expected since previous studies showed that less that 10^{-9} M PGE2 had little effect on these differentiation antigens for MDC. Although endogenous production of prostanoids does not modify HLA-DR or the co-stimulatory molecules CD40, CD80, and CD86, COX-2 produced prostanoids do markedly modulate the expression of the maturation antigen, CD83, in sCD40L/IFN-γ stimulated MDC. It appears that the threshold for prostanoid regulation of CD83 differs markedly from that of HLA-DR and co-stimulatory molecules. In the case of co-stimulatory molecules, cells producing higher levels of

PGE2 than MDC, perhaps macrophages, within the local environment may be required to affect the upregulation of these molecules as previously described (Jonuleit *et al.*, 1997).

In this study, a divergent regulation of IL-12 by COX-2 mediated prostanoid production is described. It demonstrates that endogenously produced prostaglandins increase the IL-12p40 in I-MDC but do not stimulate IL-12p70 production. This prostanoid-mediated enhancement of IL-12p40 production by I-MDC may serve to limit the Th1 immune response as IL-12 p40 homodimers function as a receptor antagonist (Ling *et al.*, 1995; Mattner *et al.*, 1993). As MDC mature in the presence of IFN-γ the level of COX-2-mediated prostanoid production increases, which effectively suppresses IL-12p70 and p40. This is in agreement with studies of others demonstrating that addition of PGE2 to cell culture reduces IL-12 production by M-MDC (van der Pouw Kraan *et al.*, 1995; 1996). Thus, endogenous prostanoids appear to play an important role in limiting the capacity of M- MDC to become a potent Th1 promoting antigen presenting cells by down regulating the production of biologically active IL-12p70 these cells. The mechanism responsible for the interesting divergence in prostanoid-mediated regulation of IL-12 has not been defined. However, modulation of surface or nuclear receptors for PGE2, e.g. EP1, EP2, EP3 or EP4 could be responsible for these changes in the response of MDC as they mature. Preliminary studies indeed suggest (Chapter 4) that the maturation stimuli regulate EP receptors expression and this defines the response of MDC to PGE2.

Previous reports have suggested that COX-2 is important for high level production of prostanoids by particular cells types and is the form of the enzyme associated with inflammation. It is of interest that when MDC are exposed to IFN-γ in

culture that PGE2 production is enhanced and that COX-2 is the predominant isoform of the enzyme used to produce prostanoids. These findings suggest that a default setting for I-MDC and maturing MDC is to increase COX-2 mediated PGE2 production when involved in inflammation, when acting as an antigen presenting cell for established Th1 responses, or when encountering other IFN-γ producing cells, e.g. NK or NK T cells. Under these circumstances, MDC are thus programmed, via COX-2 expression, to suppress IL-12 production and thus autoregulate its capacity to further stimulate Th1 T cells.

Prostaglandin production by MDC appears to play an important and focused role in the function of MDC. From the findings of this study it appears that MDC tend to produce lower levels of PGE2 than produced by monocytes or macrophages. The lower level of prostaglandins produced by MDC may be of a practical importance as that these lipid molecules work in an autocrine fashion modulating MDC function, and perhaps in regulating T cells within their microenviroment in a paracrine fashion. Working in this manner the effects of prostanoids would be contained and limit the untoward effects of these molecules. In this context, determining the regulation of the prostanoid receptors on MDC and T cells thus becomes critical to understanding the effects of these lipid molecules on their target cells. Furthermore, the production of prostaglandin by MDC may provide these cells with a self-contained "signal 3" as proposed by Kalinski et al. (1999) which would polarize the MDC away from stimulating Th1 responses, perhaps more toward a Th2 promoting antigen presenting cell (Kalinski *et al.*, 1999a). These findings also have important implications regarding the effects of COX inhibitors, particularly the new class of COX-2 specific drugs, on the immune response. The potent

anti-inflammatory action of these drugs may in part be limiting MDC maturation. These studies also raise a potential concern regarding the possibility that COX-2 specific drugs could potentiate Th1 responses by removing the suppressive effects of prostaglandins on IL-12 production by M-MDC. Further study *in vivo* is required to establish the effect of these drugs on the biology of MDC.

CHAPTER 4
MATURATION STIMULI AND MODULATION OF PROSTAGLANDIN RECEPTORS REGULATE THE EFFECTS OF PGE2 ON INTERLEUKIN-12 PRODUCTION BY MONOCYTE DERIVED DENDRITIC CELLS

Review of Literature

Dendritic cells (DC) are potent antigen presenting cells that express high levels of MHC and B7 molecules and readily activate T cells. Following interaction with CD40L expressed on T cells, DC undergo maturation further up-regulate MHC and B7 molecules and initiate production of the cytokine IL-12p70 (Cella *et al.*, 1996). This cytokine is composed of covalently linked p40 and p35 subunits and is central to the development of a Th1 immune response (Trinchieri and Gerosa, 1996). However, when DC are resting or in an immature state, they secrete only IL-12p40 as a homodimer or monomer that functions as an IL-12p70 antagonist (Ling *et al.*, 1995; Mattner *et al.*, 1993). Thus, the capacity to regulate DC production of IL-12p70 and p40 is critical to controlling the development of Th1 responses important for inflammation, transplant rejection and many autoimmune diseases (Sutterwala and Mosser, 1999).

Prostaglandins have been identified as potent regulators of IL-12 production by monocyte-derived DC (MDC) (Rieser *et al.*, 1997; van der Pouw Kraan *et al.*, 1995; 1996). Rieser et al. showed that addition of exogenous PGE2 to MDC cultures or the addition of compounds increasing cAMP increased total IL-12 secretion (IL-12p40) in immature MDC (Rieser *et al.*, 1997). However, when MDC are undergoing maturation, prostaglandins and cAMP analogues (N6,O2'-dibutyryl adenosine-3',5'-cyclic

monophosphate) inhibit production of both IL-12p70 and p40 (van der Pouw Kraan *et al.*, 1995; 1996). In addition, the previous chapter indicates endogenous prostaglandin production by MDC stimulates immature (I-) MDC cells to produce IL-12p40 while suppressing IL-12p40 and p70 production by maturing MDC (Chapter 3). However, when MDC are fully mature, the production of IL-12p70 and p40 becomes resistant to the regulatory effects of PGE2. These studies suggest that activation/maturation of MDC causes a functional transition with regard to the action of prostaglandins on IL-12 production (Kalinski *et al.*, 1999). The factors responsible for this transition remain largely undefined.

A potential etiology for the change in response of MDC to prostaglandins could be modulation of its prostaglandin receptor as these cells mature. PGE2 mediates a wide variety of cellular responses by binding to a diverse repertoire of prostaglandin receptor subtypes on nuclear and cellular membranes. PGE2 receptors (EP1, 2, 3, 4) are members of the seven transmembrane rhodopsin-type G protein coupled receptors and are pharmacologically defined. EP1 is linked to an unidentified G protein and uses Ca^{++} as a second messenger. EP2 and EP4 receptors are coupled to a G-protein (Gs) that signals by stimulation of adenylate cyclase and increases in cAMP. Seven different isoforms of EP3 resulting from splice variants in the carboxyl terminals have been described. The major effect of signaling through the EP3 is the inhibition of adenylate cyclase and decreases in cAMP, although one isoform of this receptor increases cAMP and another mediates responses through inositol triphosphate (Narumiya *et al.*, 1999).

Although the effects of PGE2 on the immune response have been widely studied, studies examining the expression of these receptors in cells of the immune system are

limited. EP4 was recently shown to upregulated in THP-1 cells with stimulation by phorbol esters (Mori *et al.*, 1996). Eriksen et al. (1985) described the prostaglandin E2 receptors on human peripheral blood monocytes (Kd= 1.1 X 10^{-9} M and 240 sites per cell) but did not characterize the subtypes of EP receptors on these cells (Eriksen *et al.*, 1985). Functionally, Meja, Barnes and Giembycz (1997) showed that either EP2 or EP4 contributed to the inhibition of LPS stimulated TNF-α production in human blood monocytes (Meja *et al.*, 1997). There is, however, no report of the prostaglandin receptor expression on MDC.

In order to determine if modulation of EP receptors was responsible for the drastic changes in PGE2 mediated regulation of IL-12 in MDC, this study examines EP receptor expression at distinct states of maturation (I-MDC, maturing MDC, and M-MDC). The importance of these aspects of MDC IL-12 regulation by prostaglandins is discussed.

Materials and Methods

Materials

PGE2, Butaprost, 11-deoxyPGE1, 19-hydroxyPGE2, Sulprostone, AH6809, and SQ29548 were purchased from Cayman Chemical (Ann Arbor MI). AH23848B was a gift from GlaxoWellcome (United Kingdom). Forskolin, 3-isobutyl-a-methylxanthine (IBMX) and N6,O2'-dibutyryl adenosine-3',5'-cyclic monophosphate (db-cAMP) were purchased from Calbiochem (San Diego, CA).

Isolation of Monocytes and Dendritic Cell Culture Conditions

PBMC were isolated from buffy coats from one unit of whole human blood using Histopaque Ficoll (Sigma, 1.077, endotoxin tested, St. Louis, MO). Cells were washed two times with Dulbecco's PBS (DPBS), Ca++ and Mg++ free (Cellgro, endotoxin

tested) and resuspended in RPMI 1640 media with L-glutamine (Gibco, BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, endotoxin tested, Logan, UT), and 1% streptomycin, penicillin, neomycin (Sigma). PBMC were allowed to adhere for 2 hours at 37°C, 5% CO2, 100% humidity and non-adherent cells were washed away with DPBS. Complete RPMI tested negative for endotoxin (<2.0 EU/ml) (E-Toxate Kit, Sigma). Adherent cells were cultured in complete RPMI supplement with 500 U (50 ng/ml) GM-CSF (Endogen, Woburn, MA) and 500-1000 U IL-4 (R&D Systems, Minneapolis, MN) to generate I-MDC. To generate M-MDC, day 6 I-MDC were harvested, washed and replated at 3.0 X 105 cells/ml and supplemented with 1μg/ml sCD40L (gift from Immunex, Seattle WA), and/or 1000 U of human recombinant IFN-γ (Endogen, Boston, MA). Some cultures were supplemented with NS-398 (Cayman Chemical), a specific COX-2 inhibitor, or indomethacin (Sigma), a COX-1 and COX-2 inhibitor.

Isolation of Total RNA and Reverse Transcription

On Day 6 I-MDC were stimulated with the maturation stimulus listed above and harvested at various time points from 2 hours to 48 hours. After stimulation, MDC were washed with PBS and immediately frozen at –70° C. Total RNA was extracted using the High Pure Total RNA isolation kit (Boeringer Mannhiem, Indianapolis, IN) that contains a DNAase treatment step. Total RNA quantity was determined by UV spectrophotometry at 260 nm. First stand cDNA was synthesized from 250 ng of total RNA with a combination of oligo dT and random hexamers (Perkin Elmer, Branchburg, NJ) with Superscript II reverse transcriptase (Gibco-BRL, Life Technologies, Grand Island, NY).

Relative Polymerase Chain Reaction (PCR)

A constant volume of the reverse transcriptase reaction (1 μl) was used in a relative semi-quantitative PCR using β-actin as internal standard control. Because the message for the EP receptors is rare in comparison to β-actin, primer competitors to β-actin were used to ensure that amplification of the endogenous standard (β-actin) was in the same linear range as the target mRNA for EP receptors. We found that a ratio of 3:7 β-actin primers:β-actin competitors and 30 cycles of PCR was sufficient to yield similar linearity to the mRNA for the EP receptors. The PCR protocol was 30 cycles of 0.5 min at 94°C, 0.5 min at 58°C, and 0.5 min at 72°C. The primers used were as follows (size of base pairs in parenthesis): EP1 (407):5'CTTCTTGGCGGCTCTCGG. 3' AGGGTGGGCTGGCTTAGTC, EP2 (394):5'GCTGCTGCTTCTCATTGTCTCG, 3'TCCGACACCAGAGGACTGAACG , EP3 (366):5' ACCCGCCTCAACCACTCCT, 3' CCGAAAAAGGTGCAGAGCC, EP4 (334): 5' GGTCATCTTACTCATTGCCACC, 3' AGATGAAGGAGCGAGAGTGG, β-actin (638):5' ATCTGGCACCACACCTTCTA, 3' GTGTTGGCGTACAGGTCTTT, competititor β-actin: 5'ATCTGGCACCACACCTTCTAAGT, 3' GTGTTGGCGTACAGGTCTTTATT (EP2 and EP4 primers (Mukhopadhyay *et al.*, 1999)). PCR products were resolved on a 2% agarose (FMC Bioproducts, Rockland, MN) gel with ethidium bromide added (Gibco). Bands were quantitated by Stratagene Eagle Eye.

IL-12p40 and IL-12p70 Assays

MDC culture supernatants from various conditions were analyzed for IL-12p70 and IL-12p40 (gift from Dr. Maurice Gately, Hoffman Roche, Nutely, NJ), by ELISA as previously described (Gately, 1999). IL-12 was standardized to pg/ml/1 X 10^6 cells.

Measurement of cAMP Formation

The cAMP level was determined by total cAMP determination kit (Amersham). Briefly, I-MDC or M-MDC were cultured at 5 X 10^5 cells/ ml in 96 well plates. Cells were incubated at 37° C for 30 minutes in complete RPMI supplemented with 5 μM NS398 and 10 μM IBMX. Cells were stimulated for 5 minutes with various agonist/antagonist to the prostaglandin receptors. After 5 minutes, cells were lysed with lysing solution provided with the kit and total cAMP by indirect ELISA was determined. cAMP is reported in pmol/ 1 X 10^5 cells.

Quantitation of ^{3}H-PGE2 Binding on I- and M- MDC

Saturation binding studies were performed as previously described (Zeng *et al.*, 1998). Briefly, 5 X 10^5 MDC were suspended in 100 μl of binding buffer in GF/C microtiter plates (Millipore, Bedford, MA) and incubated at 4° C for 1 hour with various concentration (0-30nM) of ^{3}H-PGE2 (Amersham). Plates were washed ten times with ice cold buffer to remove non-specific binding. Filtered plates were allowed to dry for 2-24 hours and then 25 μl of scintillation fluid was added and radioactivity counted (1450 Microbeta Wallace, Trilux liquid scintillation counter, 58% efficiency). Specific binding was calculated by subtraction of nonspecific binding, suspensions containing 10^{-5}M unlabelled PGE2 (Cayman Chemical) for each concentration of ^{3}H PGE2. Saturation data were analyzed by PrismGraphpad. Competitive binding studies were performed exactly the same as described above except 10^{-9}M -10^{-4}M of unlabelled selective agonists were employed.

Results

PGE2 Regulates IL-12 in I-MDC Through EP2 and EP4 Receptors

I-MDC were cultured with defined concentrations of PGE2 and secretion of IL-12p40 and IL-12 p70 were analyzed by ELISA after 48 hours. Because we previously showed that MDC produce endogenous prostaglandins, particularly PGE2, we performed this experiment in the presence of a cyclooxygenase-2 (COX-2) specific inhibitor, NS-398. As seen in Figure 4-1a, the cyclooxygenase-2 specific inhibitor NS-398 decreases IL-12p40 production by I-MDC and replacement with nanomolar concentrations of PGE2, equivalent to the level of prostaglandin produced spontaneously by these cells, restores IL-12p40 production to baseline. As reported, we found that I-MDC do not produce IL-12p70. However, I-MDC do produce IL-12p40 which is increased in a dose dependent fashion when these cells are cultured in increasing concentrations of PGE2 (Figure 4-1a). Previous reports suggested that PGE2 stimulates IL-12 production through the activation of adenylate cyclase (Rieser *et al*., 1997). Indeed, this experiment shows PGE2 readily stimulates cAMP in I-MDC (Figure 4-1b.). In addition, when I-MDC are cultured with db-cAMP, IL-12 p40 secretion is increased to a similar degree as with 10^{-6} M PGE2 (figure 4-1c; 2 and 3). These results suggested that EP receptors that increase cAMP, most likely EP2 and/or EP4, may play a role in regulation of IL-12p40 production. To evaluate this possibility we assessed the effects of EP2, EP2/4 and EP3 specific agonists on IL-12 production *in vitro*. At the present time no EP1 or EP4 specific agonists are available. We find that the EP3 agonist had no effect on IL-12p40 production (Figure 4-1c;4). However, agonists that stimulate either the EP2 receptor (butaprost and 19-hydroxy PGE2) or both EP2 and EP4 (11deoxy PGE1) stimulate IL-12p40 production

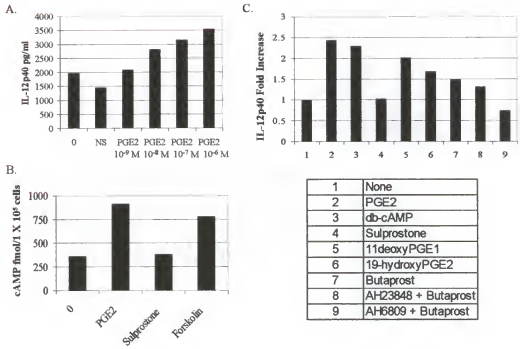


Figure 4-1: IL-12 and cAMP production after stimulation with PGE2 or prostaglandin receptor agonist in immature monocyte derived dendritic cells. (A) Immature-MDC were stimulated 48 hours with increasing doses of PGE2 in the presence of NS398, a specific COX-2 inhibitor. Supernatants were harvested after 48 hours and IL-12p40 and IL-12p70 measured. IL-12p40 expressed in pg/ml/1 X 10^6 cells. No IL-12p70 detected.(B) Total cAMP production measured in I-MDC in the presence of NS398 and IBMX, a PDE inhibitor, after 5 minute stimulation. Total cAMP reported in fmol/1 X 10^5 cells. (C) IL-12 production in I-MDC after 48 hours with the agonist/antagonist 1-9: Sulprostone, EP1/EP3 agonist; 11deoxyPGE1, EP2/EP4 agonist; 19hydroxyPGE2 and Butaprost, EP2 agonist; AH23848, EP4 antagonist; and AH6809, EP2 antagonist. Data are representative of at least three experiments and are presented as fold changes from baseline of no agonist/antagonist added.

(Figure 4-1c; 5-7). Butaprost stimulation achieves approximately 60% of the IL-12p40 levels stimulated by PGE2 and the specificity is demonstrated by the EP2 specific antagonist AH6089 that totally blocks this effect. The addition of the EP2/4 agonist 11deoxy PGE1 stimulates levels of this cytokine nearly identical to that of PGE2. These results suggest that both EP2 and EP4 receptors are involved in mediating the effects of PGE2 on IL-12p40 production with the EP2 receptor mediating the majority of the effects of PGE2 on IL-12 production in I-MDC.

Because antibodies are not available to human EP receptors, mRNA expression of these receptors on I-MDC was performed by a semi-quantitative competitive RT-PCR assay using primer sequences for human EP1, 2, 3, and 4 (Mukhopadhyay *et al.*, 1999). I-MDC do not express mRNA for EP1 and express very low levels of transcript for EP3 receptors (data not shown). I-MDC predominantly express mRNA for EP2 with lower levels of EP4 message detected (Figure 4-2).

To evaluate the expression of the PGE2 receptors on I-MDC, saturation binding studies using 0-30 nM ^{3}H-PGE2 in the presence and absence of 1000-fold excess of unlabeled PGE2 was employed. Figure 4-3a shows the saturation binding curves and Figure 4-3b, the Scatchard transformation of the saturation binding data using Graphpad Prism software. These studies reveal that I-MDC express 1087 binding sites per cell with a K_d=3.2X10^{-10}M. To determine the relative expression of EP receptor subtypes on I-MDC, competitive binding assays employing ^{3}H-PGE2 and unlabeled EP specific agonists were performed. As seen in Figure 4-3c-f, ^{3}H-PGE2 is not displaced by SQ29548, a thromboxane agonist and a negative control for EP receptor binding. However, 11deoxy PGE1, a combined EP2/4 agonist, displaces almost 100% of PGE2

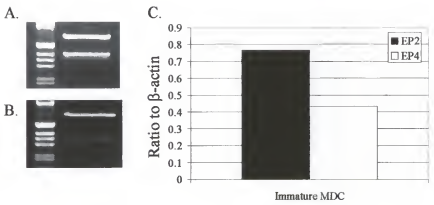


Figure 4-2: EP2 and EP4 mRNA production in immature monocyte derived dendritic cells. RT-PCR performed on day 6, I-MDC by semi-quantitative, relative PCR exactly as described in Materials and Methods. (A) β actin (638 base pairs) and EP2 (394 base pairs). (B) β actin (638 base pairs) and EP4 (334 base pairs). (C) Graphic representation of ratio of EP2 and EP4 to β actin as quantitated by band density on Stratagen Eagle Eye.

binding at 10^{-4}M, while butaprost, an EP2 specific agonist, competes with PGE2 displacing over 60% of this ligand at a concentration of 10^{-4}M. Finally, sulprostone, an EP1/ EP3 agonist, displaced little PGE2 in these studies. These binding studies are consistent with data from the RT-PCR and EP2, EP2/4 and EP3 agonist studies and suggest that I-MDC express predominantly EP2 and EP4 receptors that mediate the effects of PGE2 on IL-12p40 production.

EP2 Receptors Mediate the Suppressive Effects of PGE2 on IL-12p70 Production by Maturing MDC

Studies by our laboratory and others (Hilkens *et al.*, 1997) suggested that I-MDC undergoing maturation with CD40L/IFN-γ produce IL-12p70 and p40, however, in contrast to I-MDC, PGE2 now suppresses production of both molecules. In order to establish whether modulation of EP receptors is responsible for the switch in the effect of prostaglandins, we repeated the RT-PCR analysis of EP receptors and agonist studies as described above. First, various specific agonists were added to the culture of maturing MDC and IL-12p70 and IL-12p40 was measured in the supernatants. IL-12p70 (Figure 4-4) and IL-12p40 (data not shown) were completely suppressed by EP2 agonists and this effect was blocked by the addition of the EP2 antagonist (see figure 4-4, 6-9). These data strongly argue that the EP2 receptor is dominant in regulating IL-12 production in maturing MDC. To assess EP receptor expression in maturing MDC, I-MDC were cultured in the presence of CD40L and IFN-γ and harvested mRNA at 2, 4, 24 and 48 hours and relative RT-PCR performed. Again, we could not detect mRNA expression for EP1 and only low levels of EP3 receptor transcripts were detected in maturing MDC at any of the time points analyzed (data not shown). However, EP2 mRNA expression

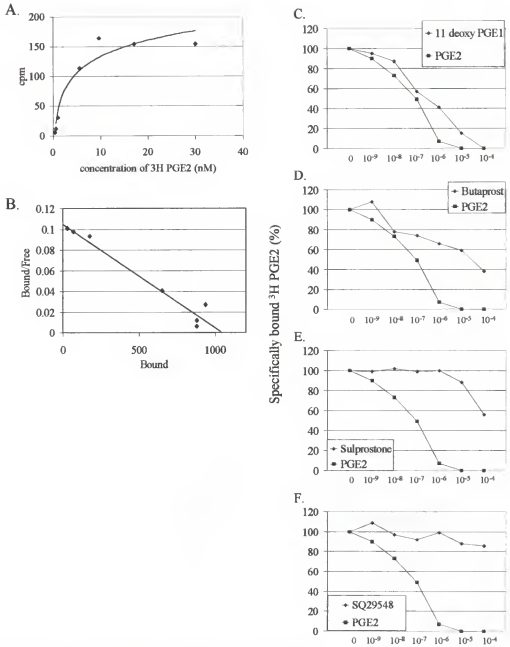
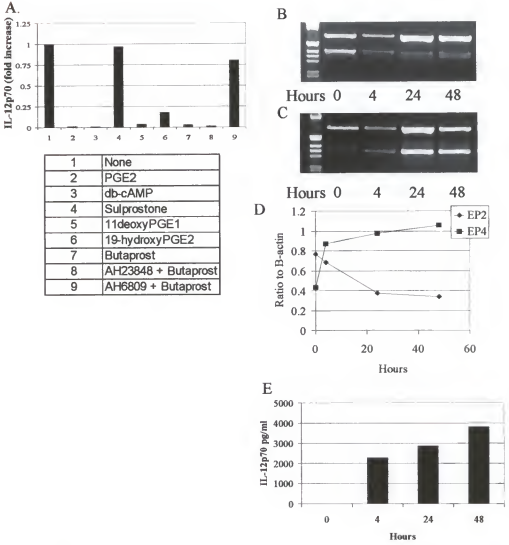


Figure 4-3: Saturation binding curves, Scatchard analysis, and competitive displacement in I-MDC. (A) Specific binding of 3H PGE2 to I-MDC. Specific binding calculated by subtracting non-specific binding, 3H PGE bound with 1000 fold excess of unlabeled PGE2, from total binding. (B) Scatchard transformation of saturation binding curve by GraphPad Prism. Bmax = 1076 sites per cells, Kd=3.2 X 10^{-10} M. Data are respresentative of three donors. Competitive displacement of specific 3H PGE2 binding (C) 11deoxyPGE1, EP2/EP4 agonist (D) Butaprost, EP2 agonist (E) sulprostone, EP1/EP3 agonist and (F) SQ29548, Thromboxane receptor agonist used as a negative control.

declined 50% over a period of 24 hours from that of the baseline I-MDC. Meanwhile, transcripts for EP4 increased rapidly within hours of stimulation and peaked almost three-fold above baseline at 48 hours of culture when the MDC is fully mature (Figure 4-4b-d). The dominance of the EP2 receptor control of IL-12p70 production was difficult to reconcile with the EP2 receptor mRNA data as it was substantially reduced during the culture period. We evaluated the possibility that the addition of PGE2 sustains the mRNA expression of the EP2 receptor. However, the RT-PCR analysis for the EP2 receptors following culture in PGE2 did not substantiate this (data not shown). These findings may be reconciled, however, if EP2 receptors are stable despite decreased transcription. Another explanation may reside in the fact that over 75% of IL-12p70 is produced within the first 24 hours of culture by maturing MDC (Figure 4-4e). Therefore, the presence of higher levels of the EP2 receptors early in the culture period is critical. These studies suggest that there are changes in the dominance of EP receptor, with the EP2 receptor playing the major role in maturing MDC while both EP2 and EP4 are important for I-MDC. However, the regulatory outcomes of PGE2 on IL-12 (e.g. up-regulation in I-MDC with down-regulation in maturing MDC) are largely governed by the maturation stimulus or by molecular changes that occur as a result of maturation.

Fully Mature MDC Express EP4 Receptors But IL-12 Production is Insensitive to the Regulatory Effects of PGE2 and cAMP

Because IL-12p70 production by M-MDC is reported to be resistant to the effects of PGE2, the regulation of this cytokine by EP specific agonists was evaluated. M-MDC were generated from I-MDC after 48 hours of culture with CD40L/IFN-γ. For the next 48 hours these cells were exposed to the same series of EP agonists as were immature and

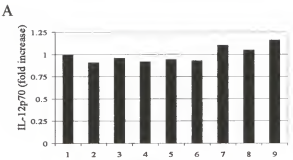


1	None
2	PGE2
3	db-cAMP
4	Sulprostone
5	11deoxyPGE1
6	19-hydroxyPGE2
7	Butaprost
8	AH23848 + Butaprost
9	AH6809 + Butaprost

Figure 4-4: IL-12p70secretion and mRNA expression of EP2 and EP4 by relative RT-PCR in I-MDC matured with sCD40L and IFN-γ. (A) Immature-MDC were stimulated with sCD40L and IFN-γ in the presence of NS398 and various agonist listed 2-9. Forty-eight hours after stimulation, IL-12p70 and IL-12 p40 were measured (IL-12p40 gave similar pattern of results, data not shown). Data are representative of at least three donors and presented as fold changes from sample with no agonist/antagonist added. Semiquantiative RT-PCR with β actin as internal standard (638 base pairs) at 0, 4, 24, and 48 hours (B) EP2 (lower band 394 base pairs) (C) EP4 (lower band 334 base pairs). (D) Graphic representation of EP2 and EP4 mRNA ratio to β actin mRNA over a 48 hour period. (E) IL-12p70 production measured at identical time points and expressed in pg/ml/ 1 X 10^6 cells.

maturing MDC. In stark contrast to the previous studies with less mature MDC, the fully mature MDC were insensitive to PGE2, EP2, EP3 and EP2/4 agonists (Figure 4-5a;5-7.). Additional studies indicated that these cells produced little cAMP in response to PGE2 or even forskolin (Figure 4-5b) suggesting a modification of EP receptors, adenylate cyclase activity or an increase in phosphodiesterase (PDE) activity. The latter would not appear to be the cause since IBMX, a global PDE inhibitor was present throughout the cAMP experiments.

To evaluate EP receptor expression on the fully mature MDC, once again RT-PCR for the EP receptors and binding studies were performed to determine cell surface receptor density and subtypes. Analysis of EP receptor mRNA expression indicated a marked upregulation of EP4 message in comparison to I-MDC and a down-regulation of EP2 message (Figure 4-4; 48 hour time point). Again, very low levels of transcripts for EP3 were found and EP1 was not expressed. Binding studies indicated that fully matured MDC express 30% fewer EP receptors than do I-MDC and have a K_d of 7.7×10^{-10}M (Figure 4-6a). Competitive binding studies suggest that the EP2 receptor is substantially reduced and that of EP4 is increased (Figure 4-6c-f). These changes in EP receptor expression could account for some of the reduced generation of cAMP as the EP2 receptors stimulates larger cAMP increases in comparison to EP4 receptors. In addition, we found a minimal response to forskolin (see figure 4-5a) suggesting decreased adenylate cyclase activity, or enhanced phosphodiesterase activity in fully mature MDC as a contributing cause for reduced cAMP generation. Finally, compounding the effects of receptor modulation and reduced capacity to generate cAMP, these experiment show that fully mature MDC IL-12 production was insensitive to db-



1	None
2	PGE2
3	db-cAMP
4	Sulprostone
5	11deoxyPGE1
6	19-hydroxyPGE2
7	Butaprost
8	AH23848 + Butaprost
9	AH6809 + Butaprost

Figure 4-5: IL-12 and cAMP production in fully mature MDC after stimulation with prostaglandin receptor agonist. Day 6 I-MDC were stimulated with sCD40L and IFN-g for 48 hours. M-MDC were harvested, washed, replated and restimulated with sCD40L/IFN-γ and the various agonist/antagonist 2-9. Data are representative of at least three donors and presented as fold changes in IL-12p70 levels from baseline (no agonist/antagonist added). (B) Total cAMP was measured in MDC matured for 48 hours in sCD40L and IFN-γ stimulated for 5 minutes with PGE2, sulprostone, or forskolin in the presence of NS-398 and IBMX. cAMP is reported in fmol/1 X 10^5 cells.

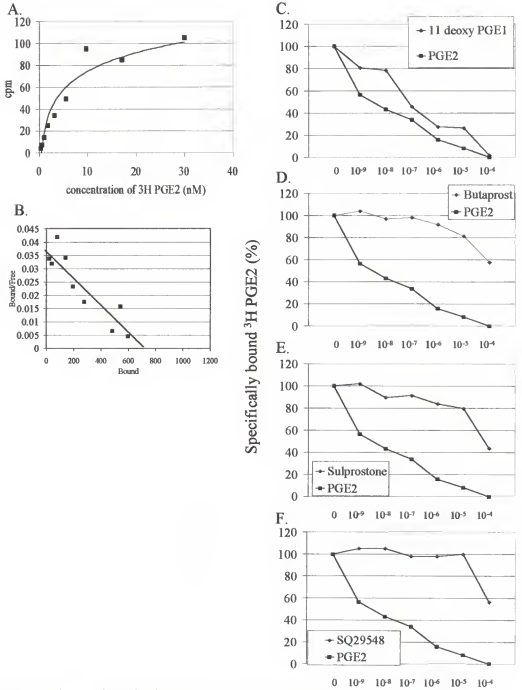


Figure 4-6: Saturation binding curves, Scatchard analysis, and competitive displacement in M-MDC. (A) Specific binding of 3H PGE2 to I-MDC. Specific binding calculated by subtracting non-specific binding, 3H PGE bound with 1000 fold excess of unlabeled PGE2, from total binding. (B) Scatchard transformation of saturation binding curve by GraphPad Prism. Bmax = 766 sites per cells, Kd=7.7 X 10^{-10} M. Competitive displacement of specific 3H PGE2 binding (C) 11deoxyPGE1, EP2/EP4 agonist (D) Butaprost, EP2 agonist (E) sulprostone, EP1/EP3 agonist and (F) SQ29548, Thromboxane receptor agonist used as a negative control.

cAMP (see figure 5a). These data suggest a series of modifications in components of the regulatory signaling pathway used by PGE2 (e.g. receptor expression, cAMP generation or stability, and cAMP sensitivity).

Discussion

These studies clearly point out differential regulation of IL-12 in MDC by prostaglandins at three distinct phases of their development, I-MDC, MDC undergoing maturation and fully M-MDC. They also for the first time characterize EP receptor by mRNA expression, prostaglandin binding and modulation of these receptors during the maturation of MDC. Collectively, these data provide a more complete understanding as to: 1) which EP receptors regulate IL-12 production, 2) how the divergent regulatory effects of PGE2 on IL-12 production are partially dictated by the dominant effect of EP2 receptors, 3) how modifications in several components of PGE2 signaling pathways ensure resistance of IL-12 to this eicosanoid or other compounds that increase cAMP, and 4) how the maturation stimulus ultimately governs the effect of prostaglandins on IL-12 production.

This study demonstrates for the first time the repertoire of EP receptor expression on human I-MDC. Based on competitive displacement studies I-MDC express EP2> EP4>>EP3 and these cells respond to PGE2 and EP2 and EP2/4 agonists by increasing the secretion of IL-12p40 with no IL-12p70 production. Stimulation of I-MDC with PGE2 or selective EP2 or EP2/4 agonists (data not shown) confirms that these eicosanoids utilize cAMP as a second messenger to mediate increases in IL-12p40. This is consistent with published results indicating that both EP2 and EP4 are G proteins that stimulate adenylate cyclase and increase cAMP (Blaschke *et al.*, 1996). The results from

our study of I-MDC and IL-12 regulation are in complete agreement with Rieser et al. (1997) who found an increase in total IL-12 when I-MDC were stimulated with PGE2, forskolin, or db-cAMP (Rieser *et al.*, 1997). The up-regulation of the IL-12p70 antagonist, IL-12p40 by PGE2 suggests that the presence of these lipid mediators may initially limit the generation of Th1 responses, or in pre-existing responses where I-MDC may be recruited, would limit this type of immune response. Recent publications indeed suggest that one of the actions of prostanoids is to down-regulate established inflammation (Betz and Fox, 1991; Snijdewint *et al.*, 1993; van der Pouw Kraan *et al.*, 1995).

The maturing MDC is complex with regard to PGE2 regulation of IL-12p70. The addition of 10^{-6} M PGE2 from the inception of culture or when added up to four hours after application of the maturation stimulus completely suppresses IL-12p70 production by maturing MDC (Jonuleit *et al.*, 1997; Kalinski *et al.*, 1997). This study determined that PGE2 suppression of IL-12p70 is mediated through the EP2 receptor and its second messenger cAMP. This regulation is somewhat perplexing as transcripts for EP2 are down-regulated by 24 hours and the majority of EP2 binding is lost 48 hours after application of the maturation stimulus. However, we find that 75% of the IL-12p70 produced by maturing MDC during a 48-hour culture is made in the first 24 hours. Therefore, the continued presence and function of EP2 receptors in the early culture period would be most critical for regulation of this cytokine.

When fully mature, MDC become highly insensitive to the effects of PGE2. We found that maturation with CD40L/IFN-γ modified M-MDC several components of the signaling pathway utilized by PGE2 to mediate suppression IL-12p70. First, this

maturation stimulus resulted in a decrease in mRNA for EP2 receptors while mRNA for EP4 receptor increased. In conjunction with these changes, PGE2 stimulation of M-MDC led to minimal increases in cAMP in comparison to much larger responses in I-MDC which express both EP2 and EP4 receptors. The limitation in cAMP production following PGE2 exposure in M-MDC may be explained by the modulation of EP2 and 4 receptors as the latter produce lower levels of cAMP when stimulated by prostaglandin binding compared to EP2 receptors (Choung *et al.*, 1998). Another explanation for decreased cAMP responses in M-MDC could be related to the described short-term agonist induced desensitization that occurs with the EP4 receptor (Bastepe and Ashby, 1999; Nishigaki *et al.*, 1998). In addition to these changes M-MDC also become insensitive to cAMP since db-cAMP no longer suppresses IL-12p70. The reasons for this loss of response have not been determined, but could be related to a loss of function of specific isoforms of protein kinase A. Overall, these data suggest several components of the prostaglandin signaling cascade are modified during maturation such that IL-12p70 production is now highly protected from PGE2 or other compounds which generate cAMP down- regulation. This feature of M-MDC may help to maintain Th1 immune responses until such time as these cells are removed or I-MDC replenish M-MDC and maturation stimuli.

In conclusion, these studies further define how PGE2 exerts diverse effects on the regulation of IL-12 in MDC at various stages of development. Because IL-12 is critical for Th1 responses, these findings have important implications for creating approaches to control Th1 immune responses and Th1 mediated-autoimmune diseases using agents such as cyclooxygenase inhibitors and selective prostaglandin receptor agonist or antagonists.

Prostaglandin receptor agonists that mediate a desired response such as decreasing secretion of IL-12p70 (e.g. butaprost) could result in desired cellular responses without unwanted affects. Given the results of this study, timing the application of these agents (e.g. early, when MDC are immature or maturing, and/or using agents such as anti-CD40 antibody to block maturation) may be critical for reducing IL-12 production. Further study is needed, however, in order to determine the effects of EP agonists or Cox-2 inhibitors alone or in combination with others to modify immune responses in the desired manner.

CHAPTER 5
GENERATION OF PHENOTYPICALLY AND FUNCTIONALLY NORMAL MONOCYTE DERIVED DENDRITIC CELLS FROM SUBJECTS AT HIGH RISK FOR AUTOIMMUNE INSULIN DEPENDENT DIABETES

Review of Literature

Autoimmune insulin dependent diabetes (IDD) results from a cell mediated response that destroys the insulin producing cells of the pancreas. Although T cells are critical to the pathogenesis of IDD, macrophages (MΦ) and dendritic cells (DC), professional antigen presenting cells (APC), are major contributors because the initiation of the autoimmune process begins with the presentation of β cell specific antigens to autoreactive CD4+ T cells as well as the important role of DC in tolerance. Defects in the stimulatory capacity of APC may promote autoimmune disease by deficient generation of regulatory cells or by impaired antigen presentation that leads to accumulation of autoreactive T cells.

Recently, Litherland et al. (1999) reported that freshly isolated monocytes from subjects at high risk for IDD aberrantly express cyclooxygenase-2 (COX-2 or prostaglandin synthase-2, PGS-2). This abnormal expression results in high levels of prostaglandin E2 (PGE2) (Litherland *et al.*, 1999). Prostaglandins, especially PGE2, have diverse effects on the immune response. PGE2 modulate T cell activation by down regulation of IL-2 production and expression of CD25 (α chain, high affinity IL-2 receptor) while promoting Th2 associated cytokines IL-4 and IL-5 (Betz and Fox, 1991; Katamura *et al.*, 1995). Additionally, PGE2 down regulates IL-12p70 production but

increases the immunostimulatory capacity of DC further modulating the response. In 1997, Kalinski et al. (1997) reported that exogenous PGE2 added to cultures of monocytes differentiating into DC affected the ability of the monocyte derived dendritic cell (MDC) to express CD1a and secrete IL-12 (Kalinski *et al.*, 1997). Additionally, Jansen et al. (1995) found that MDC from subjects with clinical IDD had reduced clustering with autologous and allogeneic T cells as well as reduced activation of autologous and allogeneic mixed lymphocyte reaction (MLR). Takahasi, Honeyman, and Harrison (1998) reported that MDC from subjects at high risk for IDD had reduced expression of co-stimulatory molecules, CD80 and CD86, and impaired antigen presentation as measured by autologous and allogeneic MLR (Takahashi *et al.*, 1998).

Because of the aberrant expression of COX-2 in peripheral blood monocytes of subjects at risk for IDD, the publish abnormalities in clustering and T cell activation of MDC in subjects with IDD, and the affects of PGE2 on MDC differentiation, this study was designed to answer the question, does abnormal COX-2 expression in subjects at risk for IDD impair MDC differentiation? Additionally, this study expands on previously published data by examining the surface marker expression, antigen uptake, cytokine production and activation of T cells by immature MDC (I-MDC) as well as MDC matured with TNF-α or soluble trimeric CD40L (sCD40L) in subjects at high risk for IDD compared to normal health controls. Using an established method for differentiating DC from monocytes (Sallusto and Lanzavecchia, 1994), this study shows in vitro generation of phenotypically and functionally normal MDC from subjects at high risk for IDD when compared to normal controls. These results may have important clinical relevance in recently proposed immunotherapy or vaccination for prevention of diabetes.

Materials and Methods

Subjects

Heparinized whole blood was collected from informed consented subjects (IRB #372-96) participating in the University of Florida subcutaneous Insulin Diabetes Prevention Trial (SQ), the Natural History of Diabetes Trial (NH) and the Diabetes Prevention Trial (DPT). Because each study had different criteria for entry, subjects were assigned risk based on the results of islet cell antibody (ICA), glutamic acid decarboxylase antibody (GAD), insulin antibody (IAA), results of first phase insulin response (FPIR) to intravenous glucose tolerance test (IVGTT), and genetic screening for the protective HLA allele DQ0602. Subjects confirmed positive for ICA and an abnormal (low) FPIR are designated high risk (HR) while subjects with a positive ICA and IAA with a normal FPIR are moderate risk (MR). Subjects with positive ICA, negative IAA and a normal FPIR are considered low risk (LR). Subjects positive for protective allele DQ0602 or who are repeat negative for ICA are not eligible and considered not at risk (NR). Heparinized whole blood from normal healthly controls was collected on the same days as blood from the subjects. Some normal control had a family history of autoimmune disease but either tested negative for ICA or had the protective allele DQ0602 and are considered NR.

Isolation of Monocyte and MDC Culture Conditions

Peripheral blood mononuclear cells (PBMC) were isolated from heparized whole blood from subjects and controls using Histopaque Ficoll (Sigma, 1.077, endotoxin tested, St. Louis, MO). Cells were washed two times with Dulbecco's PBS (DPBS), Ca^{++} and Mg^{++} free (Cellgro, endotoxin tested) and resuspended in RPMI 1640 media with L-

glutamine (Gibco, BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, endotoxin tested, Logan, UT), and 1% streptomycin, penicillin, neomycin (Sigma). PBMC were allowed to adhere for 2 hours at 37°C, 5% CO_2, 100% humidity and non-adherent cells were washed away with DPBS. Complete RPMI tested negative for endotoxin (<2.0 EU/ml) (E-Toxate® Kit, Sigma). Adherent cells were cultured for 6 days in complete RPMI supplement with 500 U (50 ng/ml) GM-CSF (Endogen, Woburn, MA) and 500-1000 U IL-4 (R&D Systems, Minneapolis, MN) to generate I-MDC (Sallusto and Lanzavecchia, 1994). To generate M-MDC, day 6 I-MDC were harvested, washed and re-plated at 3.0 X 10^5 cells/ml and supplemented with 50 ng/ml of human recombinant TNF-α (Endogen) or 1 μg/ml sCD40L (gift from Immunex, Seattle WA). Some cultures were supplemented with 5 μM NS-398 (Cayman Chemical, Ann Arbor, MI), a specific COX-2 inhibitor .

Flow Cytometry for Surface and Internal Proteins

The following monoclonal antibodies directed against surface or internal proteins were used: CD14, HLA-DR (clone L243, Becton-Dickinson, San Jose, CA or clone Tü36, Pharmingen, San Diego, CA), CD1a, CD86, CD80, CD40 (Pharmingen), CD83 (Coulter-Immunotech, Miami, FL), and COX-2 (Cayman Chemical). Appropriate fluorochrome labeled isotype control antibodies were used. Cells were suspended in PBS with 1% BSA (reagent grade, Sigma) and 0.1% Sodium Azide (Sigma). For surface marker labeling, cells were incubated with 1 μg of fluorochrome conjugated antibody/1 X 10^6 cells for 20 minutes at room temperature, then washed one time with 2.0 ml of PBS and resuspended in 500 μl of 1% formaldehyde in PBS. Intracellular labeling of COX-2

was performed as previously described (Litherland *et al.*, 1999). All cells were analyzed on Becton-Dickinson FACSCalibur or FACSort.

Autologous and Allogeneic Mixed Lymphocyte Reaction (MLR)

MDC were washed and replated at various concentrations in 96-well plates. Autologous or allogeneic nylon wool purified T cells were added at 1.5 X 10^5 cells/well. Each condition was performed in triplicate. Proliferation was measure on day 5 by a 16-hour pulse with [3H] Thymidine (1 μCi/well, Amersham Life Sciences, Arlington Heights, IL). Some autologous MLR were supplemented with GAD, tetanus or insulin peptide to measure specific T cell responses.

Measurement of IL-12 and Prostanoids

Supernatants from cultures of MDC were harvested for analysis of PGE2 and IL-12. I-MDC were cultured for 6 days, washed from the plate, counted and re-plated at 3 X 10^5 cells/ml in media containing GM-CSF and IL-4. I-MDC were cultured for an additional 48 hours before supernatants were harvested for analysis. Supernatants from maturing M-MDC, were prepared by harvesting I-MDC on day 6, re-plating these cells at the same density in media containing GM-CSF, IL-4 and maturation stimuli. Cells were cultured for an additional 48 hours and then supernatants harvested. MDC culture supernatants from various conditions were analyzed for IL-12p70 and IL-12p40 (gift from Dr. Maurice Gately, Hoffman Roche, Nutely, NJ), by ELISA in duplicate as previously described (Zhang *et al.*, 1994). The lower limit of IL-12p40 and IL-12p70 detection in this assay is 15.6 pg/ml. PGE2 and thromboxane were measured by competitive enzyme immunoassay (Cayman Chemical). IL-12, PGE2 and thromboxane values were standardized to pg/ml/1 X 10^6 cells.

Measurement of Endocytosis

Mannose receptor-mediated endocytosis was measure by the cellular uptake of FITC-Dextran (FD, 40,000 MW, Molecular Probes, Eugene, OR) and quantitated by flow cytometry (Sallusto *et al.*, 1995). Approximately 1.5 X 10^5 MDC were incubated in complete RPMI with 25 mM Hepes and 1 mg/ml of FITC-Detran for 1 hour at 0 °C and 37°C. After 1 hour, cells were washed 4 times with ice-cold 1X PBS with 0.1% Sodium Azide and immediately tested on the flow cytometer (Becton-Dickinson FACSCalibur, San Jose, CA). Fluid-phase endocytosis by macropinocytosis was measured by cellular uptake exactly as described for uptake of FD except 1 mg/ml Lucifer Yellow (LY, dipotassium salt, Molecular Probes) was used. Mean fluorescent intensity (MFI) of 37°C – 0°C was used to evaluate antigen uptake during different maturation states of MDC.

Statistical Analysis

Surface marker expression data were statistically evaluated by analysis of variance with log transformation. All other data were evaluated using two-tailed Student's t test or pair t test as indicated in the table legends. Level of statistical significance was set at $p < 0.05$.

Results

Table 5-1 details the demographics of the study set including the sex, age (mean and range) and risk of normal controls and subjects with risk for IDD. Table 5-2 shows the statistical summary for the surface markers used to distinguish DC in I-MDC and table 5-3 shows the summary for I-MDC matured for 48 hours with sCD40L, a in vitro T cell mimick. No statistical difference was noted for percent of cells positive (I-MDC

Table 5-1: Demographics of study participants.

	Number		Age		Sex		Risk^				
	Total	Sampling	Mean	Range	Female	Male	NR	LR	MR	HR	Diabetics
Controls	31	126	30.4	11 - 47	12	19	31	0	0	0	0
Subjects	86	167	22.9	4 - 76	42	44	11	37	15	19	4

^ NR= not at risk, LR = low risk, MR= moderate risk, HR= High risk based on criteria listed in Materials and Methods.

Table 5-2: Phenotypic marker expression in I-MDC from normal controls and subjects at risk for IDD.

Variable	n	Control	Subject	p ^
CD1a percent	24	82.8	70.5	0.1334
CD14 percent	24	14.1	13.5	0.8561
CD40 percent	14	95.5	92.2	0.2332
CD80 percent	16	49.0	36.3	0.4538
CD83 percent	8	5.4	3.6	0.7342
CD86 percent	16	44.9	45.3	0.6903
HLA-DR surface percent	26	91.7	86.5	0.3842
COX-2 percent	16	63.8	51.5	0.4016
CD1a MFI	24	118.0	106.0	0.7419
CD14 MFI	24	24.0	23.0	0.9718
CD40 MFI	14	68.8	55.6	0.6313
CD80 MFI	16	26.1	24.3	0.7755
CD83 MFI	8	4.7	7.8	0.2571
CD86 MFI	16	45.1	45.5	0.9165
HLA-DR surface MFI	26	178.9	258.6	0.6899
COX-2 MFI	16	29.9	28.8	0.9595

^ No statistically significant differences were noted between the two groups measured by the analysis of variance with log transformation.

only) or mean fluorescence intensity (MFI) as measured by flow cytometry and analyzed by analysis of variance with log transformation. Because of the small study size, statistical analysis between risk groups was not possible.

Because of the importance of MHC molecules in T cells activation and the reported association of HLA haplotypes and IDD, surface and total HLA-DR for I-MDC and I-MDC matured with TNF-α for 48 hours in the absence and absence of NS398 are shown in Table 5-3. Initially, a difference was noted in the MFI of total HLA-DR for TNF-α stimulated I-MDC (in the presence and absence of NS398) using the Tu36 antibody. This difference could not be substantiated with a different HLA-DR clone, L243. Both clones of antibody are documented to bind to the non-polymorphic region of the α and β chains of human HLA-DR. HLA-DR is a known risk factor for IDD. It is possible that the Tu36 antibody does not bind as well as L243 to HLA-DR molecules associated with risk to IDD. The HLA-DR types for participants of the DPT are not known; therefore, preferential binding to specific HLA molecules could not be investigated at this time.

High levels of PGE2 (10^{-6}M) inhibit the ability of the MDC to secrete IL-12. This study evaluated the spontaneous production of PGE2 in I-MDC and the secretion of IL-12 in I-MDC and I-MDC matured with TNF-α of subjects at high risk for IDD and normal controls. The results are summarized in Table 5-5. The ability to uptake antigen by receptor-mediated mechanisms by I-MDC as well as the ability to shut down the antigen uptake machinery in M-MDC was investigated. No differences noted in I-MDC or M-MDC (Table 5-6). Finally, MDC from subjects at risk for IDD and normal controls

Table 5-3: Phenotypic marker expression in M-MDC matured with sCD40L from normal controls and subjects at risk for IDD.

Variable	n	Control	Subject	p ^
CD1a MFI	8	48.1	26.8	0.16
CD80 MFI	8	49.4	41.1	0.48
CD83 MFI	8	146.2	157.4	0.82
CD86 MFI	8	681.3	600.5	0.63
HLA-DR surface MFI	8	2141.2	2064.0	0.97

^ No statistically significant differences were noted between the two groups measured by the analysis of variance with log transformation.

Table 5-4: Mean Fluorescent Intensity comparisons of Surface and Total HLA-DR in I-MDC and I-MDC stimulated with TNF-α normal controls and subjects at risk for IDD.

		L243				Tu36			
		n	Control	Subjects	p †	n	Control	Subjects	p †
I-MDC	Surface	10	237.2	257.6	0.7522	14	49.4	32.3	0.4216
I-MDC	Total	10	912.2	1042.2	0.7007	14	181.1	92.3	0.1148
I-MDC/NS	Surface	10	269.0	257.0	0.9155	14	33.1	33.3	0.9906
I-MDC/NS	Total	10	818.2	941.8	0.5736	14	125.3	71.9	0.1394
I-MDC+TNF	Surface	12	671.5	904.5	0.1740	18	78.8	43.2	0.1068
I-MDC+TNF	Total	12	2211.0	1739.5	0.3771	18	423.4	169.6	0.0151*
I-MDC+TNF/NS	Surface	10	657.6	775.2	0.5473	14	93.5	47.1	0.0932
I-MDC+TNF/NS	Total	10	2096.2	1683.4	0.3933	14	339.1	137.4	0.0124*

L243 and Tu36 are two different clones of monoclonal antibody to human HLA-DR. * Total HLA-DR shows statistically significant differences with Tu36 clone in I-MDC stimulated with TNF-α with and without NS398, a specific COX-2 inhibitor, but no differences were found with L243. † Student's t test (two tailed) used for statistical analysis with $p<0.05$ as level of significance.

Table 5-5: IL-12 and PGE2 production in MDC from normal controls and subjects at risk for IDD.

Variable	MDC Type	n	Controls	Subjects	p†
IL-12	I-MDC	23	4873.6	3101.4	0.4304
IL-12	I-MDC + TNF	19	6661.6	9463.6	0.4673
PGE2	I-MDC	17	1670.8	1485.1	0.8534

† Differences in mean values were compared by Student's t test

Table 5-6: Comparison of receptor mediated antigen uptake in MDC from normal controls and subjects at risk for IDD.

MDC	n	Controls	Subjects	p †
I-MDC	19	396.2	466.1	0.6979
M-MDC	19	50.6	123.4	0.1544

Antigen uptake measured by FITC-Dextran (mean MFI). †Student's t test (two tailed) used for statistical analysis.

Table 5-7: Mixed Lymphocyte Reaction in normal controls and subjects at risk for IDD.

		Allo	Auto	GAD	Insulin	Tetanus
	n	8	8	7	7	7
Controls	mean	2.74	4.17	1.34	1.21	1.13
Subjects	mean	3.62	3.60	0.91	0.83	0.89
	p #	0.28	0.84	0.20	0.08	0.23

Allo = allogeneic, Auto = autologous mean index shown. Index is calculated by ^{3}H thymidine incorportation of MLR divided by ^{3}H thymidine incorportation of T cells alone. GAD = glutamic acid decarboxylase, index for GAD, insulin, and tetanus is ^{3}H thymidine incorportion in antigen specific MLR divided by ^{3}H thymidine incorportation of autologous MLR. #Indices were compared by the Student's t test (two tailed) with $p<0.05$ as level of significance.

were compared in the ability to activate T cells in autologous, allogeneic, and peptide specific mixed lymphocyte reactions. Table 5-7 summarizes the findings.

Discussion

Although previous investigators have reported differences in the expression of costimulatory molecules, CD80 and CD86 (Takahashi *et al.*, 1998) and stimulatory capacity in autologous and allogeneic MLR (Jansen *et al.*, 1995; Takahashi *et al.*, 1998), this study finds no phenotypical or functional differences in monocyte derived dendritic cells for the studies performed between subjects at high, moderate or low risk for developing diabetes and controls that are at no risk. Several differences between the previous reported findings and this study exist which may account for differences in findings. First, the quantity of GM-CSF and IL-4 used in the Takahasi, Honeyman, and Harrison (1998) study was 400 U/ml of IL-4 while this study used 500-1000 U/ml. Shuler et al. (1999) reports that <200 U/ml of IL-4 give variable generation of MDC from monocytes whereas >200 U/ml of IL-4 was adequate for differentiation except in an occasional donor and 1000 U/ml of IL-4 always was successful in generation of MDC from monocytes (Shuler *et al.*, 1999). These results suggest that the level of IL-4 used in this study (>500 U/ml) may be artificially high when compared to physiological levels and the results noted between this study and the Takahashi, Honeyman, and Harrison study (1998) may explain the differences in the MDC generation. Additional studies employing varying quantities of IL-4 may be helpful in sorting out the inconsistencies.

Although the method described in this study yielded phenotypically and functionally "normal" MDC, it is possible that generation of MDC in vivo may be defective in subjects at high risk for autoimmune diseases such as IDD. Recently, a

subset of circulating, mature T cells expressing an invariant TCR α chain, referred to as NK T cells or NK1.1 in mice, were shown to be able to produce large quantities of IL-4 or IFN-γ rapidly upon interaction with CD1d molecules on dendritic cells, and may be major source of these cytokines during an immune response (Chen and Paul, 1997). Additionally, one study suggests that mice that are deficient in NK T cells naturally or by selective reduction are prone to autoimmunity (Mieza *et al.*, 1996). One study in humans, also, reports deficiency of NK T cells in humans with IDD (Wilson *et al.*, 1998). Therefore, the possibility exist that subjects at high risk for IDD may indeed have defects in differentiation or generation of MDC which were overcome as a result of the culture conditions used (high levels of IL-4).

The ability to generate "normal" MDC from subjects at high risk for IDD may have distinct advantages in using MDC as immunotherapy for prevention of IDD. The ability of producing large quantities of immature DC from monocytes has led to many studies proposing the use of dendritic cells as therapeutic agents in the treatment of tumors, allograft tolerance, and autoimmunity. The ability to generate phenotypically and functionally normal responding MDC from subjects at high risk for IDD may provide a therapeutic mechanism for induction of islet cell tolerance. Indeed, adoptive transfer studies of matured myeloid DC from the pancreatic draining lymph node of NOD mice into young NOD resulted in transfer of protection from diabetes and tolerance to β cell antigens (Clare-Salzler *et al.*, 1992). Additionally, Shinomiya et al. (1999) showed that DC from the spleens of non-diabetic female NOD mice matured in the presence of IFN-γ prevented diabetes by an unknown mechanism. They suggest that the age of the recipient and route of entry of DC are important for the anti-diabetogenic capacity of the tolerizing

effect (Shinomiya *et al.*, 1999). Others have suggested that genetically engineered DC such as DC that express immunosuppressive agents such as IL-10 or TGF-β may induce a Th2 response or anergy. Alternatively, DC that express pro-apoptotic molecules such FASL may induce activation induced cell death (Lu *et al.*, 1999).

This study shows that regardless of in vivo defects associated with inability to activate T cells, generation of phenotypically and functionally normal MDC can be achieved using standard techniques. These ex vivo derived MDC may provide a mechanism for induction of tolerance against β cell antigen or other autoantigens; however, the mechanism of induction of tolerance needs to be elucidated.

CHAPTER 6
SUMMARY AND CONCLUSIONS

This study has examined the role of prostaglandins, especially PGE2, in the maturation and function of MDC. Dendritic cells are the most potent antigen presenting cells and are unique in their ability to activate naïve T cells. Because of their critical role in the adaptive immune response, their use in immunotherapy for treatment of tumors, transplantation, vaccines and autoimmunity have been proposed. Understanding agents that modify DC which subsequently alter the T cell activation process may provide means to manipulate DC in vitro to achieve desired response in vivo.

Chapter 3 describes the constitutive expression of COX-2 in MDC and the production of prostaglandins that autoregulate their maturation and secretion of IL-12, a critical proinflammatory cytokine. Endogenously produced MDC prostaglandins do not contribute to the differentiation of MDC from monocytes as measured by standard surface marker expression (e.g., CD1a, CD14, CD40, CD80, CD83, CD86 and HLA-DR) but do contribute to the expression of CD83, a mature DC specific marker, in MDC matured with sCD40L and IFN-γ. The secretion of IL-12 in I-MDC and M-MDC is regulated by endogenously produced prostanglandins; however, the effect is opposed. Immature DC that only produced IL-12p40 decrease secretion in response to blocking endogenous prostaglandins while M-MDC that produce IL-12p40 and IL-12p70 increase secretion of both forms when COX-2 mediated prostaglandins are inhibited. These

results provided the basis for examination of prostaglandin receptors as a mechanism for divergent regulation of IL-12 secretion in I- and M-MDC.

Chapter 4 focuses on the role of prostaglandin receptors as mediators of IL-12 regulation in I-MDC and M-MDC. This study examines EP1, EP2, EP3 and EP4 in I-MDC, maturing and fully matured MDC by competitive RT-PCR, ^{3}H-PGE displacement with EP specific agonists, and measurement of IL-12p40 and p70 after exposure to receptor specific agonists. I-MDC predominantly express EP2 and EP4 receptors and when stimulated with EP2/EP4 agonists or cAMP analogues increased IL-12p40 two-fold. In the presence of CD40L/IFN-γ, the maturing MDC produces IL-12p70 and p40 that are completely suppressed by EP2 agonists and cAMP analogues. During MDC maturation, EP2 mRNA gradually declines by 50% over 24 hours while EP4 mRNA increases rapidly by two-fold at 4 hours and remains increase after 48 hours. After 48 hours of stimulation, MDC are fully mature and express 30% fewer prostaglandin receptors and EP4 is dominantly expressed. Despite expressing EP receptors, IL-12p70 production by fully mature MDC is completely insensitive to PGE2 as well as forskolin or cAMP analogues. These studies demonstrate three diverse responses of MDC IL-12 production, corresponding to distinct maturation states and characterize the role of specific EP receptors in each response. It is apparent from these studies that EP2 and EP4 receptors play a dominant role, however, the presence of the maturing stimulus reverses the effects of the EP2-mediated signal from stimulatory in I-MDC to suppressive in maturing MDC. These studies also demonstrate that full maturation alters several components of the PGE2 signaling pathway such that MDC IL-12 production is well protected from prostaglandin-mediated suppression.

Finally, studies described in Chapter 5 compared MDC from subjects at high risk for IDD and normal controls. Previously described aberrant expression of COX-2 in monocytes from subjects at high risk for IDD and effects of PGE2 on MDC differentiation from monocytes, prompted this investigation. Although no significant differences were noted between subjects with high risk for IDD and normal controls with respect to typical DC surface marker expression, production of cytokines, antigen uptake and ability T cell activation, this study provides a springboard for future studies including the use of autologous MDC for induction of tolerance to β cell antigens in subjects at high risk for IDD.

Several problems were encountered during these investigations. First, in any human based study, the person-to-person differences, whether it is number of molecules per cell of a specific cell surface marker or quantity of cytokine secreted, vary tremendously and make between groups comparisons hard to interpret. Because of the variability, sometimes displaying the data as "representation" instead of combining many experiments by calculating means and standard error better characterizes the observable result. Second, in the study comparing MDC from subjects at high risk for IDD and normal controls, the amount of sample received on each subject and control was limited; therefore, not every variable was tested on every subject or control. This resulted in a small sample size for each variable. The small number of subjects for each variable made stratification of risk groups (HR, MR, LR) impossible to analyze statistically with any power and, perhaps, gave inaccurate conclusions when risk groups were lumped together as "subjects". In retrospect, fewer variables and combination of risk group (for example,

NR with LR and MR with HR) may have increased the sample size for each group and provided enough numbers to stratify the analysis such that differences could be realized.

There are three potential areas for future experiments involving comparisons of MDC from subject at risk for IDD and normal controls. (1) Differentiation of MDC using varying amounts of IL-4 to determine if a threshold for IL-4 is required for differentiation of MDC from monocytes. It is possible that monocytes from subjects require a higher units of activity of IL-4 that normal controls. This could explain differences in the results of this study and previous studies. (2) Ability of the MDC to shut down receptor mediated antigen capture machinery in MDC matured with TNF-α. Although no statistically significant difference between normal controls and subjects at risk for IDD was noted in the uptake of antigen as measure by FITC-Dextran in I-MDC, the ability to shut down receptor mediated endocytosis in the M-MDC matured with TNF-α approached statistically significance ($p=0.1544$) and with a larger number of samples and risk stratification, it may be possible to detect differences. (3) The differences detected in total HLA-DR, but not surface HLA-DR molecules, on MDC from subjects at high risk for IDD and normal controls when using one clone of monoclonal antibody, Tu36, but not clone L243, is intriguing and raises interesting questions about the structure of intracellular HLA-DR in subjects. One potential explanation that is easily answered is that Tu36 does not bind as well to intracellular DR03 or DR04, HLA types associated with risk for IDD, compared to L243. Intracellular HLA-DR of these HLA risk types compared to other HLA-DR types may provide resolution to this query.

Characterization of prostaglandin receptors on I-MDC and M-MDC is a novel discovery and opens many potential avenues of research. First, does the microenvironment modulate the expression of these receptors? In these studies only maturation stimuli was added to the cultures when expression of the receptors was investigated. Of interest, does the expression of prostaglandin receptor change when maturation or function modulators, such as IL-10 and TGF-β, are added to the culture. Second, IFN-γ is a potent activator of MDC. After encountering IFN-γ is the MDC terminally differentiated? Does IFN-γ "lock" the MDC into a Th1 versus a Th2 inducing APC? Finally, previous studies suggest that differing numbers and subtypes of prostaglandin receptors on T cells or APC modulate the effects of PGE2 and may be a potential mechanism of skewing the T cell response toward Th1 or Th2. Additionally, subjects at risk for IDD (and NOD mice) express COX-2 abnormally in monocytes (MΦ in NOD mice) and produce higher levels of PGE2 compared to normal controls (and control mice). Examination of prostaglandin receptors on activated T cells and APC from subjects with high risk for IDD (and NOD mice) compared to normal controls (and control mice) may provide differences in sensitivity to PGE2 and may provide better understanding of polarization of T cells toward Th1 or Th2.

In conclusion, these studies examine the role of prostaglandins in the maturation and function of MDC. Understanding the effects of micronenviromental factors, present during antigen capture and maturation that modulate MDC function, may provide new approaches to manipulate the MDC to 1) induce T cell activation for immunity against infectious agents or tolerance against self-peptides, 2) regulate T cell responses including polarization toward Th1 or Th2, and 3) terminate immune responses.

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